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Oh, New Delhi; oh, Geneva

HAS the presence of the World Health Organisation (WHO) in India at the Research Unit on Genetic Control of Mosquitoes, New Delhi been a serious attempt to raise the health standards of the Indian people—or has WHO been, perhaps unwittingly, a vehicle for the United States to extend its knowledge of biological warfare procedures? That question has led to some appalling misunderstandings on the international science scene, the publication of a report by the Indian government strongly condemnatory of the operation and now the entire withdrawal of the WHO from the unit. There are some harsh lessons for all concerned in the extraordinary, and little publicised, events of the last year.

The programme

Three species of mosquitoes were earmarked for attention when the unit was established in 1970; *Culex fatigans*, *Aedes aegypti* and *Anopheles stephensi*. *C. fatigans* was the first to be studied in detail; it is the most ubiquitous nuisance mosquito and the vector of filariasis in many parts of India. *A. aegypti* is less widely distributed; it is virtually absent in villages and not common in most towns. It is the only proved natural vector of urban yellow fever; it is also a vector of dengue and chikungunya. *A. stephensi* is the principal vector of malaria.

It is widely accepted that since a mosquito control programme must take note of a much wider ecosystem than simply that of the mosquito in question, it is bound to contain elements of many different control techniques. Genetic control, the most recently promising technique, started to be widely discussed in India in the early 1960s.

Genetic control may be by one of several methods, amongst which are:

- sterile male techniques: males are sterilised by radiation or chemical treatment;
- chromosomal translocations: the normal chromosome arrangement can be broken by radiation or chemical treatment—the abnormal rearrangement or translocation does not normally cause sterility, but the offspring carry the translocations and are more likely to be sterile.
- cytoplasmic incompatibility: some strains of a given species show mutual

incompatibility, and the eggs of such matings are infertile; the introduction of an alien strain thus promotes suppression of a population.

First moves to establish a joint WHO/Indian Council of Medical Research (ICMR) unit to study genetic control were made in 1969. An agreement between the WHO and the Government of India was signed in June 1969 and authorised research on the three species mentioned above.

Progress would be reviewed by the representatives of ICMR, WHO, the US Public Health Service (USPHS) and the (Indian) National Institute of Communicable Diseases (NICD). PL-480 funds from the US government would be made available, and in addition WHO would support a Project Leader, 2 other professional staff and underwrite various other expenses. The agreement was to run for six years in the first instance. Staff levels early in 1975 were: 2 foreign scientists (WHO staff), 13 Indian scientists, and 139 other Indian staff.

In the first four years, work was concentrated on *C. fatigans* partly because there had already been some success in genetic control of this species by chemosterilisation in the United States and by cytoplasmic incompatibility in Burma. Twelve villages in the vicinity of Delhi were singled out for attention, and it was hoped that they could be treated as islands with trivial migration over the kilometre or two that typically separates villages. A variety of sterilisation techniques were employed and mosquitoes were released either as pupae or adults.

Unfortunately it was soon discovered that breeding grounds for *C. fatigans* were not confined to drains and pits in the villages but were fairly uniformly distributed over the countryside in irrigation wells. Further, *C. fatigans* proved to be a good traveller; sometimes as much as 85% of the adult population of a village were immigrants from up to ten kilometres away.

Thus the field experiments, although they showed maximum sterilities on egg rafts of well over 50 per cent were somewhat disappointing.

First criticism

All was not well, however, in the public understanding of these experiments. On the one hand, it was neces-

sary to work hard at the local level to convince villagers that the release of vast numbers of mosquitoes and the associated operations might ultimately be beneficial. On the other there was evidence of some hostility to the programme at a scientific level when the *National Herald* for February 11, 1972 published an article 'Science or Neo-imperialism' by 'A Scientific Worker'. The writer declared that international cooperation in science could often degenerate into a donor-recipient relationship with little opportunity for the recipient to evaluate and influence the work, especially if the recipient nation's scientific elite "is so brain-washed that they are at ease only in the donor nation's social outlook and science programmes". This was to be exemplified by the mosquito programme, run for the benefit only of the US Department of Agriculture (USDA) which could use India as a test-bed for chemosterilisation experiments too dangerous to be permitted in the United States. For, the argument ran, the unit should have tried the three sterilisation methods of irradiation, incompatibility and chemicals and chosen the most appropriate, but it had not purchased a cobalt-60 source necessary for irradiation, nor had it pursued much research on incompatibility. The emphasis on chemosterilisation was inevitable, continued the article, since the first two project leaders were from the USDA with long experience of and a continuing enthusiasm for chemical methods.

This was slightly odd, as two of the five field experiments already performed had used irradiation techniques and a large experiment just about to be performed was to use the incompatibility technique.

The newspaper report went on to describe the chemical used (thiotepa) as a modification of mustard gas, producing 'mutations, cancer and foetal deformities in experimental animals.' In one experiment "50,000 mosquito pupae, dipped in the dangerous chemical for 3 h and washed with water, were placed in all the wells of a small village. No one bothered how much residue of the dangerous chemical absorbed or sticking to the insects could directly pollute the drinking water." This is at variance with the unit's own version of these experi-



ments. They claim that drinking water wells were never used, only between six and ten disused irrigation wells; cans of the sterilised pupae were floated in the experiment in question, and in later experiments suspended above the water level. They further claim that chromatographic analysis showed an extremely rapid breakdown of thiotepa residues in the body of the mosquito (half-life, 6 hours).

The unit's recently issued version of these experiments should not go entirely unchallenged. Dr R. Pal who was with the unit during the experiments and is now at WHO headquarters wrote in a review book 'The Use of Genetics in Insect Control' of the same experiment described in the *National Herald*, 'Initially mortality of the released pupae was heavy due to release into the village well water' (so containers of laboratory water were used). Nonetheless, the implication behind the article was that this was the way things were always being done whereas it is clear that the unit's techniques evolved quite rapidly; indeed by the beginning of 1972 pupae were to be used no more.

Even though the article could be faulted as erroneous scaremongering with a touch of anti-Americanism added in, it carried one clear message—that someone close to, perhaps even within the unit was seriously dissatisfied. No doubt it put the unit on its toes to ensure that its future operations could not be faulted; this was to have unfortunate consequences.

More recent work

Work on *Aedes aegypti* at the unit started much later and never reached anything like the intensity of work on *C. fatigans*. After two preliminary studies in Delhi in which laboratory strains were successfully incorporated into the normal population, a more extensive study was done in Sonapat City in 1973 along the same lines. There had been considerable optimism that *A. aegypti* would yield definitive results more quickly on the feasibility of genetic control methods since the densities of the mosquito are generally lower than those of *C. fatigans*, and *A. aegypti* has more limited ability to migrate. Genetic control experiments were planned for February 1975 but, as we shall see, were not carried out.

Work on *Anopheles stephensi* was even less advanced; it was only in 1973 that a scientist was hired specifically to work on *A. stephensi*, but the intentions since then have been to increase emphasis on the species in the unit. First field trials were planned for 1975.

New criticism

Work at the unit was struck a major blow by the appearance of a newspaper article on July 29, 1974 entitled 'WHO

works for US secret research in India' and written by Dr K. S. Jayaraman, Science Correspondent of the Press Trust of India. The report was fiercely critical of three operations; the other two were the Bombay Natural History Society's bird migration study and a malaria eradication programme in Jodhpur. Dr Jayaraman wrote of tight secrecy in the mosquito project. Some experts, he claimed, believed data could be useful in germ warfare, or that India was being used as a guinea pig "for chemicals or methods not permitted in sponsoring countries." The experiments "appear to have no relevance to malaria and filariasis." Instead the unit was collecting data on *A. aegypti*, a vector of yellow fever—of which "there had not been a single case in India for ages." *Aedes aegypti* could be very useful in transmitting viruses "because its eggs (unlike those of other mosquitoes) can be dried, put on a piece of paper in an envelope and mailed to any part of the country where they can hatch." Yellow fever was believed to be absent in India because of natural cross protection from another virus. "Experts ask if genetic mosquito experiments would affect or remove this natural cross protection. If it does, yellow fever would strike India."

The story had evolved in an interesting way. When Dr Jayaraman first started to enquire about the unit in April 1973 he met with some reluctance from its then project leader to speak in other than generalities, owing to the unfavourable publicity received in 1972. A new project leader, Dr G. Brooks, said in December 1973 that he would get clearance from WHO in Geneva for a more detailed briefing, but clearance never came. Meanwhile the West German embassy in Delhi published, in February 1974 a magazine *German News* in which were printed the views of Professor H. Laven, an insect geneticist from Mainz and regular consultant to the unit. Laven who had worked for many years on incompatibility techniques, was critical of the chemosterilisation experiments which, he declared, had left the mosquito population unaffected. Jayaraman confronted Brooks with Laven's statement and as a result the Press Trust of India carried both Laven's article and Brooks' short rejoinder. But Brooks was still unable to get WHO clearance for more detailed information to be given to Jayaraman.

Jayaraman then contacted Dr R. Pal the Indian malariologist who was responsible for the unit's programme at WHO's headquarters. Pal told Jayaraman that he could give no more details of the operation as it was 'sensitive to the Indian press', presumably as a result of the earlier *National Herald*

article. At about this time, Jayaraman alleges, he was indirectly sounded out for a job as an information officer at WHO headquarters.

Response

There were two major consequences of the publication of Jayaraman's article—scientists rapidly reviewed the programme, and a rather slower political investigation was set up. The scientific and administrative review was conducted under the auspices of ICMR. There was obvious concern that the WHO/ICMR agreement needed some tidying up to strengthen Indian involvement in the management of the unit. In particular the consultative role of the Indian counterpart originally provided for in the agreement to assist the project leader needed to be taken more seriously. The scientific review committee, consisting of twenty-one members, could find little wrong with the unit's operations, however, and congratulated the unit on its achievements. Its only recommendation of substance was that an independent monitoring body should be established to oversee future releases of genetically manipulated mosquitoes.

The perspicacious reader of this complicated saga will recognise at this stage all the ingredients to ensure that the political investigation would be explosive:

- a complex scientific investigation with, like all such, its failures and blind alleys as well as its successes
- genuine and sincere disagreement amongst scientists about the correct procedures
- a journalist kept at arms length
- an agency in Geneva suspicious of the Indian press
- US involvement both through money and personnel
- a scientific review that seemed to close ranks.

The consequence was almost inevitable.

It was helped by the way that the Public Accounts Committee went about its business; it indulged in what the Americans would call a fishing expedition. Everything that could possibly be found against the project or those involved in it was brought out—from a contravention of the Registration of Books Act, in not printing the publisher's name on leaflets distributed to villagers, to doubts on Dr Brooks' abilities as project leader as he had only obtained his PhD in 1973 (though by then he had worked as a public health entomologist for 16 years).

In January 1975 the committee heard representatives of ICMR and NICD and of the Ministries of Health and Family Planning, Defence and Agriculture, of whom technical and administrative questions were asked. The com-

mittee then examined Dr T. Ramachandra Rao, a distinguished entomologist who had worked closely with the unit since his retirement as director of the Virus Research Centre, Poona. Finally, the committee in March 1975 took evidence from Dr Jayaraman and his Editor-in-Chief Mr C. Raghavan. The report was published in late April. **Were they fair?**

It is perhaps not insignificant that nowhere in the report is the unit given its correct title by the committee or the journalists—it is always referred to as the Genetic Control of Mosquitoes Unit, the word 'Research' somehow being lost; perhaps also lost at the same time was some understanding of the complex, frustrating, and often illogical nature of research. It is perhaps also not insignificant that Mr J. Bosu, chairman of the committee had already sufficiently made up his own mind, even before hearing the journalists (who were the only witnesses to level accusations of biological warfare), to write to Mrs Gandhi in late January accusing the US of using the programme, first, to carry out harmful experiments in India which would not be permitted in the US, second, to make preparations in case the US ever wished to wage chemical, bacteriological and virus warfare against India, and third, to prepare for such warfare using India as a base.

It also seems unfortunate that the committee should have delayed hearing the journalists who were presenting, as it were, the case for the prosecution until after they had heard all other witnesses. Dr Jayaraman and Mr Raghavan presented an imposing and detailed case but no-one was then asked to review this case scientifically.

One is forced to the inevitable conclusion in reading the committee's report and particularly its recommendations that its mind was clearly made up that the unit was involved in nefarious activities, and that the testimony of the journalists was only needed to provide confirmation. But the journalists' evidence needed the most detailed point-by-point investigation. For example although the journalists took a swipe at almost everything in sight, their central argument could probably be summarised as that the unit was devoting an unreasonable amount of its effort to *A. aegypti* which could be used as a vector of yellow fever. For instance, Mr Raghavan said 'An analysis of GCMU's (*sic*) activities in the last five years suggests that GCMU is primarily interested in the collection of data on the ecology and dispersal of Indian mosquitoes, particularly *Aedes aegypti* which is a vector of yellow fever.' The remark necessarily went unchallenged and in its conclusions the committee spoke of the unit's

'preoccupation' with *A. aegypti*. But if the committee had even bothered to do a simple thing like count up the number of papers published by the unit in its past five years, it would have seen the *C. fatigans* papers outnumbered *A. aegypti* papers by a ratio of four to one.

Likewise assertions that India's immunity to yellow fever might be weakened by experiments eliminating *A. aegypti* were not confronted with elementary arguments such as that *A. aegypti* already does not exist at all in many Indian cities and nearly all villages—indeed it was eliminated from Poona in 1953 without any incidence of yellow fever.

Biological warfare

Since the committee preferred the journalists' views to those of Dr Rao and the Ministries on all matters of dispute it was easy for it to follow them in the step from believing that the unit was preoccupied with *A. aegypti* to believing that preoccupation with *A. aegypti* meant preparation for biological warfare. It is not clear that the committee believed that any of the foreign scientists working at the unit were witting tools of the US Department of Defense, but it was certainly convinced that evil purposes lay behind it all. After all, the committee was told that the US Public Health Service (which releases PL-480 funds to WHO) had been known to receive money from the US Biological Warfare Research Centre at Fort Detrick (for a study of a fungal disease in 1967) and there were other links including 'efforts to avoid duplication'.

The committee were also told that the Stockholm International Peace Research Institute (SIPRI) had reported that biological warfare could be conducted through infected mosquitoes. (It was not told that it is only the females that pick up and transmit viruses, whereas the unit's field work has been exclusively concerned with males.) The committee concluded 'it is likely that the ultimate and only beneficiary of the GCMU (*sic*) experiments is the US military machine . . . The benefits, if any, that are likely to occur to India are not immediate but only potential.' The last sentence needs no comment. WHO has now pulled out.

We would be the first to admit that the thought of the US government using an intermediate organisation to learn how to infect Indians with yellow fever is utterly revolting. We would also admit that in military matters, particularly those of espionage, the standards of proof of involvement have, of necessity, to be lower than a judge, or a scientific referee, would deem adequate. But in this case the chain of logic is tenuous in the extreme: Fort Detrick has been known to collaborate with the US Public

Health Service; the USPHS represents the United States in dealing with WHO; WHO does some research in India on *A. aegypti* males; *A. aegypti* females could transmit yellow fever; yellow fever could be a biological weapon against India; therefore Fort Detrick is using the WHO to study the possibility of biological warfare in India. Perhaps the committee have more compelling evidence than they have printed. If so they should publish it at once.

That an enquiry could be conducted in this way should be a matter of concern to Indian scientists. For not only did the committee seem prepared to look for the worst in everything, but also if a spirit is abroad which looks with suspicion on anything with the remotest possible military connection, then India will find few opportunities to collaborate with the rest of the scientific world. Almost everything has its military aspect, and for good measure the committee suggested intense scrutiny headed by the Scientific Adviser to the Ministry of Defence on collaboration in

- oceanography
- meteorology
- remote sensing
- microbiology, epidemiology, ecology and virology
- toxicology
- propagation of radio waves
- science in border areas, such as Himalayan geology.

But WHO equally responsible

If one cannot but deplore the way in which WHO has had to pull out of such an important project and leave it to ICMR as best it can afford to carry on, the blame is really WHO's as much as anyone's. If it had not been for the totally leaden-footed way that the organisation has behaved, the unit would still be alive and well. It has shown itself completely incapable of handling public relations in a way to prevent a relatively small matter inflating to gigantic dimensions. In 1973 it should have pursued a policy of being open with the Indian press—open about failures as well as successes; once-bitten-twice-shy is not a philosophy to adopt towards the press. And yet even in late 1974, when *Nature* carried a report of the fuss that had been created by Dr Jayaraman's article, we received no spirited defence of the unit from Geneva. Only now is it likely that WHO will defend the unit publicly. Did WHO believe that it shouldn't get involved in squabbles in the press? Obviously WHO has political problems in dealing on a country-by-country basis with controversy. But if it cannot be more nimble in dealing with criticism, the organisation's influence is bound to diminish. □

international news

THIS has been a long, hot summer for the National Science Foundation (NSF), the agency chiefly responsible for supporting basic research in the United States.

For several months, NSF has been the target of mounting criticism from some members of congress, who have accused it of, among other things, wasting taxpayers' money on trivial research projects and of corrupting children's minds by supporting the development and marketing of controversial school science curricula. Such attacks are nothing new—in fact, they have been breaking out sporadically since the mid-1960s. But this time, instead of petering out, the criticism has lately turned into a broad assault on the entire process by which NSF determines which research grants to support, and which to turn down. Some issues of vital importance to university scientists are involved.

The matter finally came to a head during the past two weeks when NSF officials were called before a subcommittee of the House of Representatives to listen to, and answer, a string of complaints about the manner in which NSF operates the so-called peer review system. Peer reviews, the process in which grant proposals are assessed for scientific merit by scientists outside the NSF, is used in some shape or form by virtually every government agency which supports academic research. It has never lacked critics, but the system has always been stoutly defended as the best, and probably the fairest method available for judging the relative merits of competing grant proposals.

NSF itself handles some 21,000 grant proposals each year, less than half of which are funded. Nearly three quarters of the proposals are sent out by mail to several scientists to review, and about a third of those are also reviewed by a panel of scientists at a meeting. The rest are reviewed by a panel only, frequently with the grant applicant along to explain and discuss his proposal. In 1974, each proposal on which NSF took action received an average of 6.4 reviews by scientists outside the agency.

Peer review will undoubtedly survive this latest assault, but it is already clear that some important changes will be made by NSF in the next few months. The nub of the matter is how secret the system should be. NSF's critics have been arguing for complete

NSF grants system under attack

by Colin Norman, Washington



Conlan: chipping away

openness by insisting that peer review reports and the reviewers' names should be made public, while NSF officials maintain that the system will break down unless some degree of anonymity can be guaranteed to the reviewers. The arguments have a familiar ring to the editors of learned journals.

At present, it is NSF policy that grant applicants can be given only paraphrases of the reports of scientists who review their proposals, but, at a meeting last month, the National Sciences' Board, NSF's governing council, decided that beginning next year, verbatim copies of the entire peer review reports will be made available to grant applicants on request. The board suggested, however, at least for the time being, that the names of the reviewers should be kept secret, and that the reports should not be given to a third party.

The new policy should at least

ensure that unsuccessful grant applicants will be informed of the chief reasons why their grant proposals were turned down, but the move certainly hasn't stifled the criticism. That was plainly evident when NSF officials and their critics met at the witness table during the congressional hearings last week.

The hearings began with a long, vituperative attack on NSF by John B. Conlan, a Conservative Republican from Arizona who has been chipping away at NSF for months because of its sponsorship of a few controversial school science courses. Calling the peer review process "an incestuous buddy system that frequently stifles new ideas and scientific breakthroughs, while carving up the multi-million dollar federal research and education pie in a monopoly game of grantsmanship," Conlan argued that the secrecy of the system makes it prone to abuse. It is a "completely arbitrary system that is closed and unaccountable to the scientific community and to the Congress," he said.

His line of attack was that NSF officials have too much power in deciding which projects should be funded, and that unless peer review reports are made available outside NSF, their decisions cannot properly be checked. "It is common knowledge in the scientific community that NSF programme managers can get whatever decision they want out of the peer review system to justify their decision to reject or fund a particular proposal," he said.

Citing some of his now unsuccessful attempts to obtain peer review documents relating to a science education course which he called "a new height in science porno literature," Conlan argued that the peer review process is "a sick system crying out for reform." His suggested reform is simple enough: "reviews and reviewers' names must be available to principal investigators and to the Congress. And a credible system must be devised for selecting peer reviewers that will completely divorce programme managers from the suspicion — sometimes warranted — that their own biases, prejudices, and special relationships dominate grant award decisions."

The demand for total openness in the system didn't go down all that well with members of the sub-committee, and it was strongly resisted by NSF witnesses. Apart from the traditional

argument that if reviewers cannot be guaranteed anonymity, they will either be reluctant to participate in the review process, or they will mute their criticisms, James Symington, the sub-committee chairman, suggested that such an open system would be potentially subject to political manipulation. "I believe that you would have a near 100 per cent adversary process generated by what you are suggesting," he told Conlan, and he pointed out that if the reviewers' names are made public, they could be subject to pressures, perhaps even from congressmen representing grant applicants in their districts.

The next critic of NSF to take the witness stand was Robert E. Bauman, a Conservative Republican congressman from Maryland, who earlier this year succeeded in persuading the House to pass an amendment to an NSF Budget Bill, which would enable Congress to review all NSF grants before they are awarded, and veto those it doesn't like. Though the fate of that amendment was uncertain at the end of last week—it was passed by the House but not by the Senate, and no compromise has been reached on the matter—the fact that it was approved by the House is a fair measure of the hostility toward NSF which has been building up in that august body.

Bauman last week argued that NSF's granting system is open to several criticisms. First, NSF has been supporting some trivial research projects which are a waste of taxpayers' money. Second, its work often overlaps with that of other agencies, and there could well be wasted money on duplicated studies. And, perhaps most important, it tends to stifle creative, but unorthodox views. Bauman claimed that he has received several letters which suggest that "the availability of NSF grants gives the stand-pat scientific establishment much more money and power to suppress unorthodox ideas than has ever been the case before." He suggested that more Congressional review of the agency is in order, and that "the peer review system must be opened up to the light of day."

Symington asked Bauman if he would provide the committee with copies of the letters containing criticisms of NSF. Bauman said he would be happy to do so, but he had some reservations about putting his correspondents' names in the public record. The irony was not lost on the committee.

NSF director H. Guyford Stever and deputy director Richard C. Atkinson then took up NSF's side of the case. They came armed with reams of computer information about how NSF's peer review system operates (see box), and argued that the process "is working with a considerable degree

One of the central complaints about NSF's Peer Review system, which has been raised in the past few months, is that the system is simply an "old boy" network which is strongly biased in favour of the large, elite universities. The suspicion, raised chiefly by Senator William Proxmire, is that the prestigious institutions not only get more than their fair share of grants, but they also provide more than their fair share of grant reviewers.

In anticipation that such complaints would inevitably surface during the hearings on Peer Review which have been taking place before a House sub-committee during the past two weeks, NSF deputy director Richard Atkinson arrived at the hearings armed with a computer analysis of NSF's granting decisions in 1974. The figures both support and refute the critics' assertions, depending on how they are interpreted.

They show, for example, that grant applicants from institutions ranked in the top 20 by the American Council on Education stand a better chance of success than their colleagues in less star-studded universities. But that is not too surprising since the institutions are considered prestigious because they supposedly have a more innovative and distinguished faculty. Atkinson noted, moreover, that "the NSF data clearly indicate that proposals submitted by scientists from the top 20 departments have the same distribution of reviewers as proposals from other schools. The assignment of reviewers in terms of the eminence of the university with which they are affiliated is not statistically correlated

with the eminence of the school from which the proposal originates." Similarly, there is no correlation between the geographic locations of reviewers and the scientist submitting the proposal.

NSF also took a look at the distribution of its funds between the states, to see whether the spread of funds is correlated with such measures as population, numbers of scientists, average income and so on. It found that three States—California, Massachusetts and New York—get more than their fair share of NSF funds, according to those criteria. Since those States contain such elite institutions as the University of California, Stanford, Harvard, MIT and Columbia, it can be argued that there is obvious bias in the system.

But, if the distribution of funds and reviewers is assessed against traditional measures of scientific excellence, such as the number of members of the National Academy of Sciences, or the numbers of former NSF graduate award holders in each State, then California and Massachusetts can argue that they are being discriminated against.

"Obviously NSF's distribution of funds turns out to be something of a compromise between a state's population and its collection of scientific talent," Atkinson noted last week. He added however that NSF has no precise formula for making this compromise—rather the various forces operating on NSF have defined this policy.

The policy will, of course, be viewed differently by different people.

of effectiveness." Stever conceded, however, that "it is true that it is possible to find specific cases where there are problems."

Stever noted that one reason why the peer review process is now coming in for considerable criticism is that "the absolute level of basic research activity supported by the federal government is inexorably declining" as the research budget is eroded by inflation. The upshot is that good grants are being turned down. "Let me ask you rhetorically," Stever said, "what is the reaction of a highly competent scientist, who intuitively knows that the proposal he submitted would produce well done, imaginative research, the results of which almost certainly would be a useful contribution to an advance in his discipline, when he subsequently is declined? True, he knows that the competition is rougher than it used to be, but he still cannot help but harbour the suspicion that something went wrong in the decision-making process."

NSF's new policy of making peer review reports available to grant applicants should help to remove some of that suspicion, particularly if unsuccessful applicants are given a chance to rebut criticism which they feel is either biased or invalid. But NSF will clearly find that it will be difficult to explain why grant applications with generally favourable reviews have been turned down. Atkinson noted that "NSF will have to document its decisions more carefully in order to explain to the scientific community why high quality proposals are not funded. This will be costly and will bureaucratise the decision process," he suggested, "but it may be unavoidable given the attitudes of suspicion and distrust that exists in the country today."

Be that as it may, there's clearly going to be a continuing debate about whether or not NSF should take the next step in opening up its proceedings, by making the names of peer reviewers public as well. □

ENVIRONMENT minister Anthony Crosland cancelled the Channel Tunnel project last winter, yet an advisory group headed by Sir Alec Cairncross (Master of St. Peter's, Oxford) and originally briefed in April 1974 reported on the economic prospects of the Chunnel only last week ("The Channel Tunnel and alternative cross Channel services"). The expense of completing the assessment and publishing the report so many months after the horse had bolted would be justified if there were anything conclusive about its findings—its remit was amended to provide guidelines for assessing cost benefit if the project was revived, but Mr Crosland in the Commons on publication day turned that down emphatically.

The report, however, cannot say "indisputably" that a Chunnel would be "better than the expansion of existing services" to meet the certain growth of traffic, especially passengers and cars. This is partly through the lack of reliable data on the Tunnel-London rail-link, and British Rail is further criticised for lack of imagination both in planning with Continental partners a high-speed alternative to the air-linking of London and Paris and over devising connections with the inter-city services within Britain. In addition, it has failed fully to exploit its cross-Channel ferry services—both conventional and hovercraft, the two profit-makers on BR's otherwise red balance sheet.

Previous studies have been unbalanced through ignoring the hovercraft factor. Hovercraft now handle 30% of the passenger traffic on the short sea routes and their potential both there and on the Channel more widely is nowhere near fully developed. The British hovercraft industry should make capital out of the report since it is currently pressing for some firm orders from British Rail (Seaspeed) for a second generation of cross-Channel hovercraft to meet the needs of the 1980s onwards. Moreover if this move is turned down there may be a permanent loss of lead in hovercraft technology (and ultimately manufacture) abroad. In other words, British Rail may be operating a Channel hoverferry service with either French or American craft in five years' time. Chauvinism has no part in the argument but good sense and timely investment have, and at the present time Britain's hovercraft industry and expertise is recognised as a European even a Western asset and brings in good export money from Iran and other OPEC areas. Big hovercraft for open sea performance is where the technological growth and profits reside, so a good deal more than an alternative to a nineteenth century engineering

fantasy hangs on the £5 million decision sought this summer.

● The Rothschild customer-contractor principle consecrated in the Government's 1972 Policy paper "A Framework for Government R & D" removed substantial sums from the direct control of the research councils to 'contractor' departments as commissioned research. Inevitably the research councils most affected—ARC, MRC and NERC are being obliged to hawk

Round Britain



their wares now to attract commissions for the work they want to do or even to complete research programmes half done. A bold example was the recent attempt by the director of the MRC Blood Pressure Unit (Glasgow) to pressurise the Department of Health through press promotion to set up a massive screening programme for people at risk from heart attacks in middle life. A two-hour MRC press briefing the day before the MRC specialist panel met to extend the current MRC funded pilot screening project for a final year described the programme and its achievements in great detail, and discussed the possibilities of a nation-wide screening programme costed at £6 million over 5–6 years. It turned out that it was not in MRC's power to take up this programme but the Department of Health could, when the pilot project was complete. If and when they do, they are likely to lean heavily on the Glasgow Blood Pressure Units' unique expertise.

● The year ending in March 1975 was one of adaptation to altered circumstances rather than of any great progress for the Medical Research Council. That is the main message of the MRC Annual Report which has just been published by HMSO. Adaptations have consisted of the post-Rothschild administrative variety, internal changes necessitated by the Council's recent reorganisation and, perhaps most importantly, changes imposed by the financial difficulties of the universities.

Total expenditure by the MRC last year, including £5.5 million coming by way of the Health Departments and the Department of Employment, amounted to £36 million. This represented a reduction of 1.7% in real terms compared with the previous year. An oddity of the financial outgoings of the MRC was that less was spent

on grants last year than expected. That was due partly to unusually late applications and partly to a growth in unfilled positions for research assistants in universities. The dual-support system of funding university research has been under great pressure with the universities increasingly unable to provide their share, namely the salary of the principal investigators, laboratory space and ordinary departmental overheads. Sir John Gray, secretary of the MRC, stated his recognition that the situation was bound to deteriorate further but added that he felt the MRC had sufficient flexibility to cope, partly by implementing a modest capital development programme for university research.

The MRC Research Groups are one casualty of the present stringencies. In the past they were set up when a university wished to pursue a particular line of research and agreed to assume financial responsibility for the Group after a limited initial period of MRC support. Universities can no longer afford to give undertakings like that and no new Research Groups were established last year.

The past year has also seen a new career structure for the scientific staff of the MRC. With less numerous and flexible careers available in universities, the MRC has been persuaded to offer far greater opportunities of tenure within its own establishments, whilst regretting the consequent reduction in the mobility of its scientists. The Cell Biology and Disorders Board of the MRC has set up two sub-committees to review specific fields of research. One, under Professor W. F. Bodmer, will be looking at the field of clinical genetics including the possibilities for screening programmes. The other, under Professor D. C. Phillips, will review molecular biology with particular emphasis on the role of the Laboratory of Molecular Biology in Cambridge. And the Council is continuing to evaluate last year's Neuberger report on Food and Nutrition Research.

● THE Committee of Vice-Chancellors and Principals introduced the first report of a study group on post-graduate education—more in the nature of a Green Paper than a White one, floating ideas for discussion in the universities in place of a hardcore of recommendations intended as policy. Nothing highly controversial in the group's findings, except perhaps a suggestion that overseas students should be asked to pay more for education at British universities than native scholars. When the government first introduced differential fees in 1966, most universities were reluctant to make the charges, and some flatly refused, but there have been changes in the status

INDIA has forced her way out of the exclusive smallpox club. From June 30, there was not a single indigenous case of smallpox in the country, according to figures received from the field from all sources including the World Health Organisation (WHO). This has been hailed as a remarkable feat. Only last year, nearly 200,000 cases with more than 30,000 deaths were reported from all over the country. Six states—Bihar, Uttar Pradesh, West Bengal, Assam, Orissa and Madhya Pradesh—accounted for nearly 99% of the cases, with Bihar leading them all with a staggering 68% of the total.

Starting in October 1973, concerted and intensive efforts were undertaken jointly by the government of India, the state governments, and the WHO to tackle the disease. Health workers literally visited each and every village in the endemic areas to inquire about smallpox cases, and as a result of hard and dedicated work, in less than two years, the disease has been wiped out.

Congratulating the workers and agencies whose efforts made this achievement possible the Union Health Minister, Dr Karan Singh, also announced that the vaccination programme would be continued for another two years to ensure against recurrence of the disease. Unfortunately, smallpox has not yet been controlled completely in neighbouring Bangladesh and there is always the danger of its importation from across the border. The government, Dr Karan Singh emphasised, was well aware of this and had already stepped up vigilance arrangements on the border with Bangladesh, so that no smallpox cases entered the country undetected. India is self-sufficient in the production of the preventative smallpox vaccine and has already donated 275,000 ampoules to Bangladesh; more will be made avail-

able to that country, if necessary.

Meanwhile, the Health Minister also announced that the award for reporting of any fresh incidence of smallpox from July 1, 1975 would be Rs.1,000—ten times what it used to be.

● India's first satellite, Aryabhata, has been orbiting the Earth for over three months now. The data being received indicate that all systems and instru-

Indian diary

from Narendar K. Sehgal

ments of the satellite are healthy and functioning normally. The three scientific experiments on board had to be switched off, however, after the first five days, following detection of a fault in one of the four lines delivering power to the experimental packages.

Scientists at the Indian Scientific Satellite Project at Peenya (near Bangalore) say they have good data from two of the three experiments—the one looking for X rays in space and the other aimed at detecting high-energy neutrons and gamma rays at times of intense solar activity—received during the first five days, but none from the aeronomy experiment which was to look for electrons in the ionosphere and ultraviolet radiation in the night sky.

As soon as the fault was detected, all scientific experiments were turned off for fear that the trouble might spread to the remaining three power lines as well. Using simulation techniques, the scientists have been working overtime to determine and pinpoint exactly what went wrong. So far, they have not succeeded. Redundant instrument capacity was not built

into the experimental packages for several reasons. The principal one was that the experiments were only a secondary objective of the Aryabhata mission. The primary aim was to establish satellite fabrication capability and to see if the various systems and components aboard functioned and performed as planned.

Indian space scientists have not given up all hope yet; they are planning to turn on the experiments again, hoping that the fault—possibly due to some stuck relay which prevents power flow in one of the lines—may have corrected itself. They do admit, however, that chances of this happening are rather slim. In the meantime, studies will continue unabated to determine the exact cause of the failure.

As if to counter this hitch, there has been an unexpectedly happy development too. The operational life of Aryabhata was originally estimated to be about six months. That was how long the inert gas supply on board (used in the satellite's stabilisation mechanism) was expected to last. But fresh calculations, based on actual data being received, show that the gas supply would last much longer, possibly more than 18 months. This will give scientists extra time in which to determine and, if possible, correct the fault and revive the experiments.

Encouraged by the success of the first attempt, the Indian Space Research Organisation has already signed an agreement with the Soviet Union to launch India's second satellite, Aryabhata-2, in 1977-78. The second satellite is expected to be a lot more sophisticated and it will carry television camera systems to survey mineral deposits and agricultural crops, among other things. The flight model for Aryabhata-2 will actually be a modified version of the back-up model of the one now in orbit.

and capabilities of countries sending students to Britain in the interim which are likely to ensure a milder response to the Vice-Chancellors' proposal.

Ten years ago the majority of overseas students came from relatively poor countries which were in no position to provide postgraduate education themselves and the facilities provided in Britain were seen as a relatively inexpensive form of aid. But these days, says the study group, many students come from countries which in terms of national income per head of population are better off than we are, and it is clearly arguable that in these cases the fee payable should be at least a significant proportion of the cost of providing the course.

The fee would not have to be so high that it was out of proportion with similar charges in Western Europe or the US (which would leave plenty of leeway with MIT charges running at \$4,000 to \$5,000 a year), and the needs of the countries which are still underdeveloped could be met by a system of postgraduate scholarships, continuing the notion of offering development aid in the form of education. Even so, the first effect of any increase in charges would almost certainly be a fall in the number of overseas students arriving in Britain.

The universities want to keep the door open to foreign students not only because of the idea of a world academic community, visiting and returning visits internationally, but also

because there is a theory that scientists and technologists trained in Britain will one day be ordering British goods when they're sitting at the biggest desk in the headquarters of the Zambian State Uranium Corporation, or Burundi Rubber. An unfortunate flaw in the theory is that British industry doesn't seem to recognise the value of postgraduate education, with the result that in certain fields it doesn't achieve the sophistication which can be managed by countries (like Germany) which automatically look for masters degrees and doctorates in recruits to their engineering industries. A member of the Vice-Chancellors' group confessed, privately, that at CERN, for example, British loyalists were wringing their

hands and looking elsewhere for equipment, simply because we don't have the calibre of engineer to produce the sort of equipment they require.

Not surprisingly then, the study group is recommending that steps should be taken to see that British industry becomes acquainted with the meaning and value of the masters degree. Also they suggest a tightening up of the admission procedures to a PhD course, in view of justified criticism to the effect that less than ideal candidates are being accepted, and in some cases are receiving the degree. To weed out the stragglers before they have gone too far, the Vice-Chancellors will be asking universities to consider the introduction of a more formal and rigorous assessment of progress, possibly in the form of a written examination or preliminary dissertation, at the end of the first year of a PhD course. And by way of consolation for

the candidates who get over this additional hurdle in the education race, there is a recommendation that a special mark of distinction might be considered for outstanding theses. So if all goes well it will only be a matter of time before the plushest jobs are reserved for thinkers with an upper second PhD or better.

Perhaps by way of a reply to Lord Crowther-Hunt, the report steered clear of making recommendations about the relation between student places and manpower requirements. Apart from areas like medicine and law, where there is a clear connection between the number of career openings and university places, the Vice-Chancellors suggest that in general, progress to postgraduate education should be on the principle that any student who is "qualified, suitable and keen to proceed" beyond first degree level should be able to do so. ☐



THIS week we're running the first in a series of competitions designed to allow scientific minds a flight of literary fancy. Two or three weeks' leeway will be allowed for overseas entrants, but UK competitors are asked to submit their efforts within a week of publication. £10 is available for the best entry or entries.

No. 1: Dissident scientists in the Soviet Union are forced to confine their activities to the meetings of the famous Sunday seminars. A prize for the minutes (not more than 150 words) of an illicit seminar somewhere in the Western world. ☐

correspondence

Sulphones for leprosy

Sir,—If Drs. Browne and Davey (*Nature*, May 15) agree with my statement (March 20) that "sulphones provide a cheap and practical form of treatment", why are most of the World's leprosy patients not receiving it? In countries or areas adopting the outpatient approach, the percentage on treatment is very high; for example, in an area of Northern Nigeria where I worked about 90%; in a remote area of New Guinea 100% (*Med. J. Anat.*, 1, 1262, 1971) and in an area of India, 60% after only three years of starting outpatient clinics (*Dan. Med. Bull.*, 20, 198, 1973). In countries persisting with institutional care, such as Tanzania, only 33% receive treatment, and some patients have to travel up to 250 miles to receive it. Rehabilitation, including reconstructive surgery, prostheses, physiotherapy and occupational therapy, has greatly magnified the cost of institutional care. Vast sums have been spent on a rehabilitation centre (ALERT) in Ethiopia for crippled patients, yet only 40% of patients are receiving dapsone. Thus, it is the strategy employed which determines the number on treatment and not economic reasons or social aspects of the disease. Will Mr Van Den Wijngaert (May 22) tell us how much of the £4 million collected by the European anti-leprosy Association (ILEP) in 1973 was spent on institutional care, including rehabilitation, and how much

on outpatient treatment?

The figures I quoted showing the rapid fall in leprosy incidence in areas where sulphones have been used, have been ignored by Drs. Browne and Davey. LEPROA have not published any incidence figures in their Malawi project, which started nine years ago, and ILEP have not used money for this purpose. Thus, it is a failure of evaluation, rather than a failure of sulphones to rapidly interrupt transmission, which is the problem. A proved and practicable method of prevention is available and no more time should be wasted on BCG or chemoprophylaxis. Immunoprophylaxis using dead *M. leprae* from infected armadillos, as suggested by Drs Browne and Davey, has no proven preventive effect.

If government are informed of these facts, if voluntary agencies spend the money on treating patients before they become crippled and if WHO arranges for proper evaluation, then all leprosy patients can be treated and the disease could probably be eradicated within the next ten years.

C. L. CRAWFORD

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North to Alaska

SIR,—In her correspondence "North to Alaska" (May 29) Angela Croome states that no ship got through the North

West passage until 1944. This is not quite correct. During the years 1903–06 the Norwegian explorer Roald Amundsen succeeded in getting his little ship *Gjøa* through. Thus he is rightly credited with the "discovery" of the North West passage.

Yours faithfully,

K. STENSTADVOLD

University of Trondheim, Norway



A hundred years ago

THE recent French inundations have recalled to memory an experiment which was tried twelve years ago before Napoleon III. The design was to manufacture mattresses of cork, so that any one on board a ship or in a house which could be flooded would have in his bed a ready-made raft capable of floating under a weight of more than 1 cwt. for any length of time. Cork is a material so soft that mattresses made of it are not inferior to any other for comfort.

from *Nature*, 12, 280; August 5, 1875.

news and views

Rhizobium as a free-living nitrogen fixer

from John Postgate

SCIENTISTS interested in biological nitrogen fixation have for many years been perplexed by the apparently complete dependence of *Rhizobium*, the bacterial partner in the well-known legume symbiosis, on its host plant. Despite frequent attempts nobody, until about six months ago, had successfully obtained growth and fixation by rhizobia in the absence of living plant material (either as a nodulated plant or an association between rhizobium and a tissue culture of plant callus). The plant, indeed, contributes genetic information important to the nodule symbiosis, so Dilworth and Parker (*J. theoret. Biol.*, **25**, 208; 1969) earlier suggested that some of the genes determining rhizobium's ability to fix nitrogen (*nif* genes in geneticists' shorthand) were actually 'banked' with the host plant. More recently evidence had been accumulating to support the contrary view: that rhizobia possess the complete quota of *nif* genes themselves but that the genes are silent in free-living cells.

One line of evidence stemmed from the work of Dunican and Tierney (*Biochem. biophys. Res. Commun.*, **57**, 62; 1974) who took a strain of *Klebsiella aerogenes* which did not normally fix nitrogen, transferred genetic material from *Rhizobium trifolii* to it, and obtained progeny which fixed. The presumption was that the *K. aerogenes* Nif⁺ progeny were using rhizobial *nif* genes but, as the authors recognised, ability to fix nitrogen is common among klebsiellae and the possibility that *K. aerogenes* possessed cryptic (latent) *nif* genes, and that some activator had been transferred, was not excluded. Circumstantial evidence came also from H. J. Evans's group, who showed that *R. japonicum* grown in conventional laboratory culture, in the absence of plant material, possess not only the electron transport factor associated with nitrogenase (*Plant Physiol.*, **51**, 136; 1973) but also a protein immunologically similar to the molybdenum-iron protein of nitrogenase (*Biochim. biophys. Acta*, **381**, 248; 1975). Even stronger evidence was provided by the experiments published in *Nature* a few months ago (see *Nature*, **253**, 305; 1975) in which

strains of the slow-growing cowpea type of rhizobium were shown not only to fix nitrogen in the presence of callus from non-leguminous plants, but also to continue fixing nitrogen for up to twelve hours after the callus was removed. Callus was clearly providing a diffusible material which permitted nitrogen fixation by the rhizobia; it might be a genetic activator, but actual gene transfer was hardly likely, particularly since non-leguminous callus functioned as well as legume callus in the artificial symbioses.

In the event, the situation has proved remarkably simple. In this issue of *Nature* three groups, two from Australia and one from Canada (pages 406, 407 and 409) simultaneously report successful cultivation of nitrogen-fixing rhizobia in the complete absence of plant material. As with the earlier experiments using callus culture associations, the possibility that the rhizobial cultures might be contaminated by free-living nitrogen-fixing bacteria had to be rigidly excluded, because rhizobia are difficult to sustain as pure cultures, and the slow growth of the cowpea group makes them even more awkward in this respect. In addition, positive acetylene tests needed to be backed up with ¹⁵N₂.

These things have been done and the key to the question proves to be the carbon source: for fixation, a pentose such as arabinose or xylose as well as a dicarboxylic acid such as succinate seem to be essential. Note that both classes of carbon source are common plant constituents. A relatively small amount of fixed nitrogen (such as glutamine, glutamate or nitrate) seems to be helpful; in this respect the cowpea rhizobia seem to resemble free-living aerobic nitrogen-fixing bacteria such as *Derxia gummosa* or *Mycobacterium flavum* which also fix nitrogen best when 'kicked off' with a little pre-fixed nitrogen. In *D. gummosa*, the need for fixed nitrogen can be simply interpreted: nitrogen fixation is an oxygen-sensitive process, so a little pre-fixed nitrogen permits colonies or cultures to grow to a density such that, within the colony, the cooperative respiration of the bacteria lowers the oxygen tension to a level at which fix-

tion can occur (Hill, *J. gen. Microbiol.*, **67**, 77; 1971).

So far, all the successful experiments with rhizobia have made use of media set with agar, on the surface of which the colonies of rhizobia grow and within which colonies the oxygen tension may range from zero to atmospheric. Since fixation by rhizobia has long been known to involve aerobic metabolism, it is tempting to assume that the cowpea rhizobia, like *D. gummosa* and *M. flavum*, are bacteria which become microaerophilic when fixing nitrogen. There is an apparent paradox, brought out by the Canadian group, that nitrate or ammonia, which repress nitrogen fixation in liquid cultures of ordinary fixers such as clostridia, azotobacters or klebsiellae, actually promote fixation by free-living rhizobia, but the local ammonia or nitrate concentration near an agar colony cannot readily be assessed and it would be premature to conclude that regulation of *nif* in these rhizobia is unusual. Resolution of both of these problems must await the successful culture of nitrogen-fixing rhizobia in homogenous liquid media, where both oxygen and fixed nitrogen concentrations can be measured. No doubt this is only a matter of time.

The substantial advance represented by this work is not only the final proof that cowpea and some other rhizobia carry the complete complement of *nif* genes; it is also the fact that many strains and species of rhizobia now join the ranks of free-living nitrogen-fixing bacteria, with revolutionary consequences for the study of their biochemistry and genetics—for one thing, the host plant can be by-passed in the laboratory. A well established obligate symbiosis is crumbling; in what does the host specificity of the traditional legume symbioses reside? And is the nodule nothing more than a compartment to restrict access of oxygen to rhizobia? If only a pentose and a dicarboxylic acid are needed for rhizobial fixation, how readily can this information be used to set up new associations with plants and forage crops? And how many other free-living nitrogen-fixing bacteria have been missed by microbiologists because two carbon sources are needed?

Degrees of antiquity

from Peter D. Moore

PRIMITIVE things have a fascination all of their own; and when those primitive things, are, in addition, both rare and attractive, it is natural that they should become a source of attention. All of these things are true of the cycads, which, being the sole survivors of a group that was widespread and successful during the Mesozoic era, were romantically termed 'living fossils' by Chamberlain in 1935. Although they are not considered to be in the direct line of Angiosperm evolution, they probably represent an offshoot from it and it is natural that modern taxonomic techniques should be brought to bear upon the problems of their internal and external relationships.

There are three extant families of cycads, the Cycadaceae, Stangeriaceae and Zamiaceae, and views have differed regarding their relative primitiveness. Sporne (*Morphology of Gymnosperms*, Hutchinson, London; 1965) considered *Stangeria* to be most primitive in that it possesses the largest number of fern-like characters, whereas Marchant (*Chromosoma*, 24, 100; 1968) preferred to regard *Cycas* and *Microcycas* as primitive, both on morphological and cytological grounds. In his cytotaxonomic work on cycads, Marchant examined chromosome number and form in 35 species belonging to eight of the ten extant cycad genera. He found that many species possess telocentric chromosomes (that is, having a terminal centromere), a character which he regarded as primitive. High chromosome number he also considered a primitive feature. On this basis *Microcycas* (Zamiaceae; $2n=26$, 22 telocentric) and *Cycas* (Cycadaceae; $2n=22$, 20 telocentric) emerge as particularly primitive. *Stangeria* (Stangeriaceae) has a chromosome complement in which $2n=16$, two only of which are telocentric. Marchant, however, admitted that it was impossible to refute the reverse argument, that telocentric chromosomes represented a derived, or advanced condition. The extreme antiquity of the genome, of course, makes interpretation difficult since there has been ample time for complex evolutionary processes.

Dossaji, Mabry and Bell (*Biochem. System Ecol.*, 2, 171; 1975) have now made a chemotaxonomic approach to the problem of evolutionary relationships among the cycads by analysing biflavonoids from their leaves. Fourteen of these compounds, which are restricted mainly to gymnosperms, were identified in the 82 species studied. Ten species of *Cycas* were uniform in their biflavonoid patterns and contained some biflavonoids, such as hinokifla-

vone, which are not found in the other two families. Within the Zamiaceae, the pattern is slightly more diverse, and both of these families have biflavonoids in common with *Ginkgo biloba*, another surviving member of a primitive group of gymnosperms. Perhaps the most striking result, however, is the complete absence of biflavonoids from the leaves of *Stangeria*. Dossaji *et al.* regard this as a derived condition in which the capacity for biflavonoid synthesis has been lost as has happened in the Pinaceae. Thus, in spite of its primitive morphology, *Stangeria* is beginning to achieve the status of an advanced cycad.

It would have pleased Chamberlain to know that it was plants collected by himself and now cherished by Professor Bold in Texas which provided the incentive for these studies.

Mapping viral integration sites with somatic cell hybrids

from J. K. McDougall

CELLS transformed by viruses into a potential or actual malignant state can usually be shown by molecular hybridisation techniques to retain at least part of the viral DNA molecule covalently linked to cellular DNA. One of the phenotypic changes which these altered cells exhibit is the expression of antigens which are virus specific, for example the tumour antigens (T-ag) and transplantation antigens (TSTA) found in cells transformed by Simian Virus 40 (SV40) and adenoviruses. The progress made in the identification of sequences of viral DNA which persist in single or multiple copies has been spectacular since the restriction endonucleases became available. Having reached a point where the viral DNA integrated into a particular cell line can be accurately described, the tumour virologist is that much closer to analysing the role of viral genes in inducing and maintaining transformation. The location(s) at which viral sequences are integrated into host chromosomes may be of prime importance in this analysis.

Croce and his co-workers (Croce, Girardi and Koprowski, *Proc. natn. Acad. Sci. U.S.A.*, 70, 3617; 1973) made an encouraging start to answering the question of whether specific integration sites exist, by making use of gene mapping techniques in somatic cell hybrids (Weiss and Green, *Proc. natn. Acad. Sci. U.S.A.*, 58, 1104; 1967). Hybridisation of SV40-transformed human cells with thymidine kinase negative mouse cells resulted in selection of hybrid cells in which reten-

tion of human chromosome C7 and expression of SV40 T-antigen, TSTA and rescue of defective virus were concordant (Croce, Huebner, Girardi and Koprowski, *Virology*, 60, 276; 1974). Fusion of mouse peritoneal macrophages, which do not divide in these *in vitro* conditions, with the virus-transformed human cells resulted in cell lines which were all transformed and which all contained C7 (Croce and Koprowski, *J. exp. Med.*, 140, 1221; 1974).

Thus the criteria for gene assignments to chromosomes (Ruddle, *Nature*, 242, 165; 1973) appear to be satisfied, allowing Croce and Koprowski (*Proc. natn. Acad. Sci. U.S.A.*, 72, 1658; 1975) to conclude that the gene(s) responsible for transformation and the integrated SV40 genome are located on C7.

The study has been taken a step further (Croce, Aden and Koprowski, *Proc. natn. Acad. Sci. U.S.A.*, 72, 1397; 1975) by injecting the transformed hybrid cells into nude mice, which act as convenient culture hosts for heterotransplants (Rygaard and Povlsen, *Acta Pathol. Microbiol. Scand.*, 77, 758; 1969). Inoculation of an uncloned population of hybrid cells, which all contained chromosome C7 together with different frequencies of 13 other human chromosomes, resulted in the development of tumours. Analysis of the tumour cells showed 100% retention of C7 and expression of SV40 T-antigen, but a reduction, often complete, in the frequency of all other human chromosomes. Although most cloned hybrid cells and the tumours derived from them were near tetraploid for the mouse and contained on average three C7 chromosomes, more recent studies (Croce, Aden and Koprowski, *Science*, in the press) have shown that hybrids with a near diploid complement of mouse chromosomes and only one C7 are tumorigenic and have the SV40-transformed phenotype.

The hybrids derived by Croce do not follow any pattern which would be compatible with the hypothesis of chromosomal balance and suppression of malignancy (see Jones, *Nature*, 252, 525; 1974). Neither the transformed phenotype *in vitro* nor tumour induction is associated with the loss of specific mouse chromosomes in the man-mouse hybrids or of human chromosomes in hybrids between normal human and SV40-transformed human cells (Croce, Huebner, Girardi and Koprowski, *Cold Spring Harb. Symp. quant. Biol.*, 39, 335; 1975). In these experiments it would seem therefore that the transformed phenotype and malignancy are under positive control.

The results from Croce's studies all show that SV40 DNA integrates into

only one chromosome, in the two biochemically abnormal human cell lines examined. No SV40 DNA sequences could be detected using the sensitive DNA-DNA reassociation kinetics method, in any hybrid lacking C7. Results from molecular hybridisation studies on SV40-transformed mouse cells (Botchan, Ozanne, Sudgen, Sharp and Sambrook, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4183; 1974) show that the pattern of viral DNA sequences integrated in transformed cells can be very complex, in that different regions of the virus genome may be present at different frequencies. The situation with adenovirus-transformed cells is similar (Gallimore, Sharp and Sambrook, *J. molec. Biol.*, **89**, 49; 1974). Kelly and Sambrook (*Cold Spring Harb. Symp. quant. Biol.*, **39**, 345; 1975) suggest an integration of SV40 DNA sequences in more than one chromosome although expression, in terms of T-antigen and cytochalasin B susceptibility, is associated with specific chromosomes. Many SV40-transformed cell lines have been shown to contain multiple copies of the viral genome (Ozanne, Sharp and Sambrook, *J. Virol.*, **12**, 90; 1973). This is again true of adenovirus-transformed cells, although the whole adenovirus genome is not found.

Before the generalisation that SV40 integrates only into chromosome C7 in human cells can be made, it will be necessary to examine other SV40-transformed human cell lines and in particular, those containing multiple copies. Weiss (*Proc. natn. Acad. Sci. U.S.A.*, **66**, 79; 1970) has previously suggested a random association of SV40 T-antigen expression with human chromosomes. A crucial question is the role of chromosome C7 in the somatic cell hybrids and whether there may not be some growth advantage conferred by host genes located on this chromosome. These might nevertheless be under the control of viral genes. Croce has been unable to obtain hybrid lines by fusion of mouse macrophages with untransformed human cells.

Somatic cell hybridisation techniques, which have accelerated the mapping of genetic loci in mammalian genomes, must now be a method of choice for localising integrated viral sequences. The results from current studies on adenovirus-transformed human and rodent cell lines, in which the viral sequences have been quantitated, will be of great interest. Croce and his colleagues have demonstrated the power of this approach, the implications of which extend beyond the experimental situation. The recognition of transforming genes, whether of viral origin or not, should be possible using these methods.

Structure and functions of an early T7 promoter

from Maria Szekely

THE nature of promoter sites, defined originally as regions of DNA required for initiation of transcription, has attracted the interest of many laboratories. Attempts to isolate these sites and to determine their structure were made as early as 1972, when Heyden, Nüsslein and Schaller (*Nature new Biol.*, **96**, 9) isolated a fragment from fd DNA, the binding site of RNA polymerase. Polymerase binding sites have since been isolated from a number of different DNAs, making use of the fact that the binding of this enzyme protects the corresponding DNA fragment from nuclease digestion. The length of the fragments obtained was in most cases around 40 base pairs, although Giacomoni *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 3091; 1974) found binding sites 14 base pairs long on T5, T7 and λ DNA by using somewhat different conditions for nuclease digestion. Recent improved techniques for DNA sequencing made it possible to establish the nucleotide sequence of polymerase binding sites in fd (Schaller *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 737; 1975), SV40 (Dhar *et al.*, *Nucleic Acids Res.*, **1**, 595; 1974), lambda P_L (Maniatis *et al.*, *Nature*, **250**, 394; 1974), tyr-tRNA (Sekiya and Khorana, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 2978; 1974) and lac DNA (Dickson *et al.*, *Science*, **187**, 27; 1975).

The function of the promoter is, however, more complex than the simple binding of RNA polymerase. The functional definition of a promoter site is in fact somewhat vague, as it is not clear whether the same DNA region is responsible for the different stages in the initiation of transcription. The functions of the promoter include providing a recognition site for the polymerase, and providing an entry site where a stable 'closed complex' can be formed (Chamberlin, *A. rev. Biochem.*, **43**, 721; 1974) which is converted into an 'open complex' in which a short stretch of the DNA strands has been separated. Only the open complex is able to start RNA synthesis. The starting point of the actual transcription process is also part of the promoter. There are, however, contradictory data and hypotheses in the literature as to whether the binding and starting sites are overlapping, or whether they are far apart, thus requiring a migration of the enzyme along the DNA. The finding of Schaller *et al.* (see above) that the sequence of a strong polymerase binding site on the replicative form of fd DNA includes the 5'-

terminal sequence of the corresponding mRNA (determined by Heyden) suggested that the binding and starting sites probably overlap. Dickson *et al.* established the sequence of the whole control region of the lac operon (*Science*, **187**, 27; 1975) and determined the site of interaction with RNA polymerase by locating the nucleotide changes produced by different promoter mutants. They came to the conclusion that this interaction site does not coincide with the starting point for transcription. They propose a scheme for initiation of lac transcription according to which the enzyme moves 35 base pairs from the entry site to the start site.

The disagreement may probably be ascribed to the fact that very different approaches and some indirect methods have been applied in both the above studies. It is therefore of great interest that a direct comparison of binding and initiation sites produced more conclusive data.

A recent article by Pribnow (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 784; 1975) elucidates these problems by identifying the binding sites of RNA polymerase at an early promoter site of T7 DNA under conditions of initiated or non-initiated complex formation and by comparing the nucleotide sequence of this site with the 5'-terminal sequence of the mRNA transcribed from this promoter. Pribnow finds that the polymerase binding site is the same whether transcription is initiated or not, and that the starting point of transcription is around the centre of the binding site.

He uses specific dinucleotides to direct initiation of transcription to one or other of three early promoter sites. Specific initiation at promoter site A3 can be achieved in the presence of CpA and a very low concentration of CTP (Minkley and Pribnow, *J. molec. Biol.*, **77**, 255; 1973). Under these conditions only the first three nucleotides of the mRNA are joined together, CpApC-OH is formed, and the polymerase is 'locked' into this site. The binding site of the enzyme—the fragment of DNA protected by the polymerase—can be isolated and sequenced. If, after the formation of this complex, transcription is allowed to proceed for 10 min, RNA fragments up to 80 nucleotides long can be obtained, the first 19 nucleotides of which have been sequenced. This sequence corresponds exactly to the 3'-half of the 43 base pair long DNA fragment which binds the polymerase. The sequencing of the DNA fragment itself was done by an indirect method: a stretch of RNA complementary to the DNA fragment was synthesised by read-through transcription and isolated by hybridisation techniques and its nucleotide sequence

was determined. Essentially the same technique was used to establish the structure of the non-initiated polymerase binding site. Tight, non-initiated binary complexes of DNA and RNA polymerase were obtained in low salt media in the absence of dinucleotides or nucleoside triphosphates. The protected DNA fragment was isolated and its sequence was found to be identical to that of the initiated binding site.

It is thus clear that the binding site of the polymerase does not change when transcription is initiated and that this binding site overlaps with the starting site of transcription. Further data show that the same DNA region does not fulfil the function of a polymerase recognition site. The protected DNA fragment is unable to rebind the enzyme (in agreement with the findings of Schaller *et al.*, see above), probably because some further information, not contained in the 43 base pairs, is required for recognition of the promoter site. Still, even in this known sequence, striking homologies are found with polymerase binding sequences in other DNAs, suggesting either that this DNA stretch may also play a part in the recognition process or that the stable binding of polymerase requires the presence of a short specific sequence in all promoter sites.

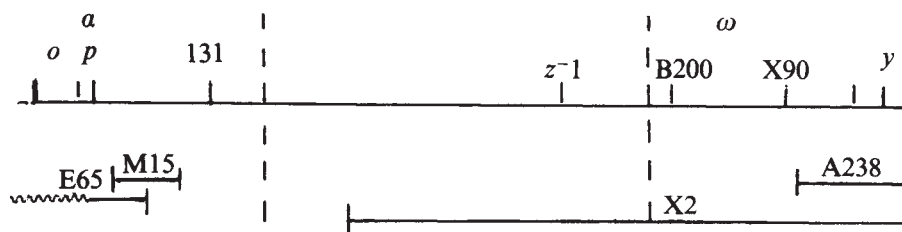
Very similar conclusions have recently been reached by Heyden, Nüsslein and Schaller (*Eur. J. Biochem.*, **55**, 147; 1975).

Protein conformation and α -complementation

from Roger Pain

RESTORATION of activity to an inactive mutant enzyme is always intriguing. When a purified system is developed such that the phenomenon can be translated from the realm of genetics into solid protein chemistry then one can expect interesting advances, both to the cell biologist and the protein conformation-activity industry.

Attention was first drawn to a particularly interesting example of mutant reactivation by Ullman, Jacob and Monod (*J. molec. Biol.*, **24**, 339–343; 1967) involving β -galactosidase, the enzyme specified by the z gene of the lactose operon in *E. coli*. Inactive mutants of this enzyme bearing mutations in a particular region of the z gene can have their activity restored by non-covalent binding of the gene product from z genes bearing mutations outside this same region. On the map of the z gene (see figure) both the operator-proximal α region and the



The z gene for β -galactosidase: figures and letters above the solid line indicate the position of point mutations in the z gene; o , operator; p , promoter; y , structural gene of β -galactosidase permease. From Ullman, Jacob, and Monod, *J. molec. Biol.*, **24**, 339; 1967.

operator-distal region specifying the N-terminal and C-terminal regions respectively of the β -galactosidase subunit can act as 'acceptors', if mutated, or as 'donors', if intact, in the reactivation or complementation process. As an example of α -complementation, the protein specified by the deletion mutant M15 can be restored to wild-type β -galactosidase activity by binding proteins arising from point mutations such as z^{-1} , B200 or X90 or deletions such as A238 or X2, but not by mutants such as 131 or E65. M15 is termed an α -acceptor while z^{-1} , A238, and so on exhibit α -donor activity. Finally, wild-type β -galactosidase consists of four identical subunits, each of molecular weight 135,000. α -complementation involves the binding of a polypeptide donor to the individual subunit, enabling it to form an active enzyme, rather than the association of different subunits; it is intra- rather than inter-cistronic in character.

A significant step forward in this fascinating phenomenon has been taken by Zabin and his colleagues who have purified the M15 protein, shown how it differs in sequence from the wild-type enzyme and also purified a sequenced peptide with α -donor activity (Langley *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 1254–1257; 1975). Cyanogen bromide cleavage of β -galactosidase yields peptides with α -donor activity; peptide CB2, residues 3–92, has been purified and found to complement purified M15 protein, giving a specific activity close to that of pure β -galactosidase. This confirms the genetic analysis that α -complementation involves the N-terminal region of the enzyme. The single CNBr peptide peculiar to M15 protein as compared with wild type β -galactosidase was found to correspond to CB2 and has been purified by an ingenious double labelling procedure and shown to differ from CB2 in that residues 11–41 are missing. It is therefore these 31 deleted residues in M15 protein whose function is supplied by the α -donor peptide CB2 in α -complementation.

An interesting model for such systems already exists in the work of Anfinsen on staphylococcal nuclease (*Science*, **181**, 223–230; 1973). In this

single subunit enzyme of 149 residues, the tryptic fragment (1 to 126)—a protein chemist's deletion mutant—can be complemented by peptide 99 to 149 with partial restoration of enzyme activity. Further, the overlapping regions may be 'trimmed' by proteolysis in the same way that certain complemented β -galactosidase mutants have been trimmed (Ullman and Perrin in *The Lactose Operon*, edit. by Zipser and Beckwith; Cold Spring Harbor, 1970). Anfinsen's well characterised model would act as a precedent for suggesting that the peptide CB2 provides residues 1–41 in the wild-type conformation with the remainder 42–92 and residues 1–10 of M15 going spare, yet without interfering with the structural features essential for activity.

The fact that M15 protein possesses a strong substrate binding site, together with the observation of Langley *et al.* that it behaves in solution as a dimer rather than a tetramer, suggests that α -complementation may be concerned with inter-subunit binding sites. The sharp delineation of the α -region shown by genetic analysis may well reflect the boundary of a protein domain such as are now known to exist in several larger enzymes. Immunological evidence in favour of this hypothesis has recently been published by Celada, Ullman and Monod (*Biochemistry*, **13**, 5543–5547; 1974).

With the ability now to study the phenomenon of complementation on purified systems it is perhaps worthwhile speculating on one or two implications for protein conformation of the original results of Ullman *et al.* Treatment with guanidinium chloride (GuCl) was found to destroy α -acceptor activity in all mutants examined, while α -donor activity was unaffected. This suggests that the N-terminal α region must be free from deletions for refolding to occur from GuCl but not presumably *in vivo*. Further, mutants having α -donor activity can be divided into two classes: mutants in the operator-distal ω region whose α -donor activity is only revealed after exposure to 6 M GuCl and subsequent return to aqueous solution and mutants in the central region between the α and ω regions whose α -donor activity is evi-

dent both before and after GuCl treatment. Consideration of the unfolding and refolding processes involved suggests that even a minimally altered ω region may affect the *in vitro* folding of other regions or domains of the enzyme in a way different from the *in vivo* situation. It must be acknowledged that these experiments were carried out on crude extracts of enzymes and mutants. If, however, they were reproduced using purified systems like those of Langley *et al.*, they would pose most interesting and potentially productive questions about the kinetic pathways along which large polypeptide chains fold in the test tube on the one hand and off the ribosome on the other.

Mössbauer effect and order-disorder transitions in alloys

from G. Longworth

To study the mechanisms involved in order-disorder reactions in alloys, the functional dependence of some physical quantity (for example resistance, magnetisation or X-ray diffraction pattern) must be measured. An ordered alloy is one whose structure may be divided into sublattices each of which is occupied predominantly by one type of atom, whereas after disordering the various atoms are arranged at random on each sublattice. Such reactions are divided into those of first or second order (degree) involving either a discontinuous or continuous change in long-range order parameter with temperature. At a first order transition, usually associated with closely packed lattices such as in Cu_3Au or Ni_3Fe , two phases of different composition may coexist, whose transformation is governed by a latent heat. The highly monochromatic gamma rays emitted by certain (Mössbauer) nuclides may be used to study order-disorder reactions by measuring the hyperfine interactions, which are a function of local atomic order.

Thus the complex ordering in Fe_3Al has been studied by Cser *et al.* (*Phys. Stat. Sol.*, **20**, 581; 1967) by determining the changes in magnetic hyperfine field (H_{hf}) at the ^{57}Fe sites. In iron alloys, H_{hf} is determined essentially by the magnetic moment on the iron atom and by the conduction electron polarisation, both of which may be a function of local atomic order. Erickson and Roberts (*Phys. Rev.*, **B9**, 9; 1974) have measured the change on ordering in the electron charge density at the gold nuclei, and in the vibrational motion of the gold atoms in Cu_3Au .

Drijver *et al.* (*J. Phys., Paris*, **35**, C6-465; 1974) have used an analysis of the hyperfine fields in Ni_3Fe to determine the long range order parameter, and more recently (*Phys. Rev. Lett.*, **34**, 16; 1975) have extended this technique to follow the time evolution of the phase transition in this alloy 'at temperature'. A detailed analysis of the Mössbauer lineshapes confirmed that the transition was first order, proceeding by way of a nucleation and growth mechanism.

In this latest work, a sharp drop in mean field H_{hf} at the iron sites at about 771 K on heating indicates a first order transition, while during cooling, the reverse path is followed with a hysteresis of about 10 K. The linewidths (Γ) at the transition were much greater than the theoretical value (0.20 mm s^{-1}), mainly due to the presence of both ordered and disordered regions having values of H_{hf} which differed by a small amount (≈ 20 koersted). The amount of ordered material at 770.9 K roughly follows the increase in the mean field $\bar{H}_{\text{hf}}$ with time, along an S-shaped curve, consistent with nucleation and growth, taking about 200 hours to complete. By comparison with model calculated spectra, it was suggested that: (1) during the ordering reaction the first nuclei present have a long range order parameter of only about 0.6. There is then a homogenous growth of order within these ordered segregates. (2) During the disordering reaction there is in addition composition segregation involving iron-rich ordered material and iron-poor disordered material consistent with a two-phase region about 4 K wide similar to that observed by Calvayrac *et al.* (*Mater. Res. Bull.*, **7**, 891; 1972) using electron microscopy. While disordering starts at a temperature at or above the two-phase region, ordering is suppressed as shown by the hysteresis and starts about 10 K below.

The disadvantage of the Mössbauer technique is that for detailed measurements above room temperature it is really restricted to iron and possibly tin alloys, since for most other Mössbauer nuclides the size of the Mössbauer effect is too small. In the case of Ni_3Fe the amount of information available from the spectra is limited by the relatively small difference in H_{hf} between the ordered and disordered phase. One might expect a larger difference when a change in crystal structure is also involved. The use of small amounts of the Mössbauer nuclide ^{119}Sn as a probe in magnetic alloys might lead to a greater change in H_{hf} , since the tin atoms carry no magnetic moment and derive their field entirely from their neighbours. Thus the Mössbauer technique is re-

stricted in its application in this field. But the advantage of being able to measure the changes in local properties at the temperatures concerned is considerable, and this technique, combined ideally with electron microscopy, could add greatly to our knowledge of order-disorder transitions in alloys.

Analysis of membrane noise

from Shin-Ho Chung

A NUMBER of researchers are using a powerful new technique to study electrical properties of nerve and muscle membranes. This technique, known as membrane noise analysis, is based on the fact that spontaneous perturbations of the mechanisms underlying membrane permeability give rise to random fluctuations in membrane conductance, and the statistical structure of these fluctuations provides, in turn, information about the microscopic events generating such noise.

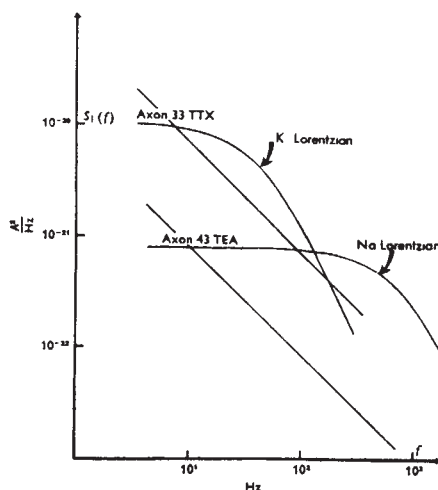
On the basis of physical theory, electrical noise arising from several different sources is expected to be present in nerve and muscle membranes. First, the thermal agitation of charge carriers will produce thermal, or Nyquist, noise. In addition, when the flow of current is due to only a relatively small number of the total available carriers (as it is in the membrane), flicker noise is shown to be present. Superimposed on these two sources, there will be random fluctuations of membrane conductance resulting from microscopic perturbation of ionic channels. For example, single ionic channels in a membrane at an equilibrium state may spontaneously open and close, although the average number of open channels remains constant. The membrane conductance will then fluctuate around its mean value. Because each of these noise sources has a different physical basis, their analysis, in principle at least, will provide answers to different sets of questions which have not previously been amenable to experimental attack. An experiment by Conti, De Felice and Wanke (*J. Physiol., Lond.*, **248**, 45-82; 1975) was aimed at elucidating some of these questions.

Two physical models for ionic channels follow from Hodgkin and Huxley's description of current flows in membranes. The first of these, a probabilistic version of the Hodgkin-Huxley equations, envisages a channel gate, guarded by four macromolecules, either in an open or closed conformation. The channel is open when all these macromolecules are in their open conformation. An alternative class of

models, based on a literal interpretation of the Hodgkin-Huxley equations, predicts that channel conduction can occur even if the molecules of the gate are not all in the open conformation. This condition allows the channel conduction to have more than just two (open and closed) states; rather, perhaps, a sufficient number of intermediate states to approach a continuum. Hill and Chen (*Biophys. J.*, **12**, 948; 1972; *ibid.* **13**, 1276; 1973) and Stevens (*Biophys. J.*, **12**, 1028; 1972) have independently derived that the spectral density for conduction fluctuations in the Hodgkin-Huxley axon (obtained from a Fourier analysis of the experimental records) obeys a mathematical form which, though not identical, resembles a simple Lorentzian function. The predicted spectrum is constant in the low frequencies and decreases according to $1/f^2$ in the high frequency limit. This holds true for both physical interpretations of the Hodgkin-Huxley equations, but the frequency at which the extrapolated $1/f^2$ decline intersects the low frequency limit is different in the two models.

Earlier measurements of voltage and current fluctuations in nerve membranes (Verveen and Dirksen, *Proc. IEEE*, **56**, 906; 1968; Poussart, *Biophys. J.*, **11**, 211; 1971) were dominated by the presence of flicker noise, and the most interesting noise, namely, that arising from ionic channels, went unnoticed. The expected Lorentzian noise from the squid axon was first detected by Fishman (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 876; 1973; *Fedn Proc.*, **34**, 1330; 1975) in his power spectrum of membrane voltage fluctuations. When tetraethylammonium ions (which are known to block potassium channels) were added to the bathing solution, the Lorentzian component disappeared, thus indicating that the detected noise arose from microscopic events associated with potassium permeability. Because the voltage noise is related to conductance fluctuations by the membrane impedance, the elementary processes of nerve excitation can be revealed more directly through measurements of current noise. Conti *et al.* (also De Fecile *et al.*, *Fedn Proc.*, **34**, 1338; 1975) have now obtained two Lorentzian components, associated with the open-close kinetics of potassium and sodium channels (see figure). From the measurements, they also derived the number of sodium and potassium channels present per unit membrane, and, from it, they calculated the absolute conductance of individual channels. Their estimate of the number of sodium channels, 330 per square micrometre, agrees well with those derived from other lines of argument (see Hodgkin, *Phil. Trans. R. Soc.*, **B270**,

297; 1975; Keynes *et al.*, *ibid.*, 365). Available data, however, are not refined enough to distinguish between the two possible physical interpretations of the Hodgkin-Huxley equations; but with luck they soon will be, as a number of laboratories are now making measurements of axon conductance fluctuations.



Components of current noise spectral density from squid axons. When sodium channels were blocked with tetrodotoxin, the spectrum consisted of the two separate components (flicker + K Lorentzian). Similarly, in an axon with its potassium channels blocked, the sum of the two components (flicker + Na Lorentzian) could adequately describe the obtained spectrum. (Modified from Conti *et al.*, *J. Physiol., Lond.*, **248**, 45; 1975.)

Analysis of noise in the muscle membrane has also provided important sets of data. One of the possible physical models emerging from such measurements is that two acetylcholine molecules binding on a receptor induce a conformational change in a gating macromolecule (Stevens, *Fedn Proc.*, **34**, 1364; 1975). The channel then allows the transmembrane flux of sodium and potassium ions. The length of time a channel stays open depends exponentially on membrane potential; for each -100 mV change in membrane potential, the lifetime of open channels doubles. At 8°C and 0 mV, for instance, a channel remains open, with a conductance of 20 to 30 pS, for about 6.5 ms. These conclusions could have been derived directly, were it possible to activate a known number of channels by iontophoretically applying acetylcholine at the endplate and, instantaneously, to cause the relaxation of the receptors back to their normal, closed, conformation. Because such an experiment is not technically feasible, quantitative information such as that obtained by Stevens can only be acquired by membrane noise analysis.

Katz and Miledi (*Nature*, **226**, 962; 1970) first discovered that a constant dose of acetylcholine applied to the frog neuromuscular junction gives rise to a substantial increase in membrane noise. In a subsequent analysis (*Nature new Biol.*, **232**, 124; 1971; *J. Physiol., Lond.*, **224**, 665; 1972), they showed that this increased noise results from endplate gating mechanisms, and calculated the size and time course of the elementary event. The technique of noise analysis was further exploited by Anderson and Stevens (*J. Physiol., Lond.*, **235**, 655; 1973). Making the assumption that the receptor molecule exists either in open or closed conformation, Stevens and his associates (see also Magleby and Stevens, *J. Physiol., Lond.*, **223**, 151; 1971; *ibid.*, 173) made a number of predictions about the behaviour of conductance fluctuations, making use of the fluctuation-dissipation theorem. The measurements of acetylcholine-induced conductance fluctuations, obtained under voltage clamp conditions, agreed well with the theoretical predictions. The striking agreement between theory and experiment does not, however, constitute final proof of the theory proposed by Stevens. In fact, a slightly different version of microscopic events, in which a channel opens instantaneously and its conductance then decreases exponentially (Katz and Miledi, 1971), will lead to the same prediction about the behaviour of conductance fluctuations. There is, furthermore, an additional observation, for which Stevens's model, in its simplest form, cannot account without making additional assumptions. This is the observation that the mean lifetime in the open state, and the absolute conductance of a single channel, can each vary when different drugs are used to open the channel (Katz and Miledi, *J. Physiol., Lond.*, **230**, 707; 1973; Colquhoun *et al.*, *Nature*, **253**, 204; 1975). The model would have predicted that a channel, once it is open, will have the same conductance whichever drug caused it to open. Nevertheless, theoretical predictions of the type Stevens has made, along with the quantitative data extracted from membrane noise analysis, will place a severe constraint on a number of tenable physical models for endplate gating mechanisms.

In the closing chapter of his classic book, *Membranes, Ions and Impulses* (University of California, Berkeley, 1968), Cole suggested that a sequel of his volume should be written in a decade or so under the title of *Membranes, Ions and Impulses: A Chapter of Molecular Biophysics*. When that chapter is finally written, we will have witnessed the final act of a long drama, staged by some of the intellectual giants

of the past and present. It is toward this goal that a group of neurophysiologists are making steady progress; and analysis of membrane noise is but one of the many tools at their disposal.

Ordered disorder?

from P. V. E. McClintock

A NEW theory of the dilute magnetic alloys known as spin glasses has been proposed by Edwards and Anderson. The burden of their argument, published in *J. Phys.* (F5, 965; 1975), is that an apparently random arrangement of magnetic spins may, in reality, sometimes be regarded as being highly ordered.

An example of the type of alloy to which their theory applies is formed when a few per cent of manganese is added to copper: the copper, being non-magnetic, serves as a relatively inert medium in which the manganese atoms are held fixed in random spatial positions. The unpaired electron spins responsible for their magnetism are, however, free to point in different directions. At high temperatures the spins will be randomly orientated with their directions changing with time, but the intriguing question arises: what will happen as the temperature is reduced?

In order to try and guess the answer it is instructive, first, to consider what happens in a pure magnetic material, for example pure manganese, where the spins are all equally spaced from each other. In such a case the mutual interactions between nearest neighbour pairs of spins would all be the same, and the system would either become ferromagnetic (all spins pointing in the same direction) or antiferromagnetic (neighbouring spins pointing in opposite directions). Which of these two types of ordered state is the one actually adopted is a sensitive function of the spacing between the atoms. Measurements of the magnetic susceptibility χ as the temperature T is reduced show that the change from the disordered to the ordered state occurs as a so-called cooperative transition, with $\chi(T)$ passing through a sharp anomaly at a critical temperature T_0 . This behaviour can be understood if one postulates a critical temperature T_0 . This behaviour spins: the larger the number of ordered spins, the larger the molecular field becomes, thus accounting for the sharpness of the anomaly and also, of course, for the description of the transition as cooperative.

On the other hand, when the manganese is diluted with copper, the magnetic atoms will no longer be spaced equally from each other, and the whole situation becomes very much more complicated. The simple mole-

cular field approach is no longer applicable because some pairs of spins will be attempting to align while others, with different mutual separations, will be tending to take up antiparallel orientations. Moreover, the different spin spacings will tend to give rise to different values of the temperature T_0 at which the ordering transition occurs. Thus, common sense would seem to suggest that any peak in $\chi(T)$ must be greatly broadened. Experiments, however, have demonstrated quite otherwise: Canella and Mydosh (*Phys. Rev.*, B6, 4220; 1972) for example, working on the somewhat similar iron-gold system, found that for an alloy containing 5% of iron there was a very sharp cusp in $\chi(T)$ at about 20 K.

To try to account for this, Edwards and Anderson have pointed out that, despite their random positioning, there must be some configuration of the orientations of the spins which minimises their potential energy. Considering the spins' positions in space to be fixed and permanent, this configuration can then properly be regarded as the ground state of the system (in fact, one of many such states, each corresponding to a local minimum in potential energy). The authors argue that the mere existence of such a state is sufficient to give rise to a well-defined magnetic ordering transition, and hence to account for the observed sharp cusp in $\chi(T)$. Thus, as T is reduced, there must eventually come a temperature at which the spins 'notice the existence' of this ground state; and, as $T \rightarrow 0$, the system will then settle into that state. Of course, if one were able to examine the material in its ground state, it would seem, superficially at least, still to be in total disarray with the spins arranged quite randomly. The randomness would be more apparent than real, however, because the system would be in the particular state which minimised its potential energy. It thus could reasonably be regarded as being, in actual fact, highly ordered.

These ideas are by no means original, other workers having previously arrived at broadly similar conclusions. The great contribution which Edwards and Anderson make in this paper is that of devising a theoretical framework within which the magnetic behaviour of such systems may be treated quantitatively. They describe the degree of order in the spin system by a parameter q which represents the probability that a spin pointing in a particular direction at one moment will be pointing in that same direction when viewed again a long time later. Thus at $T=0$, where the system is magnetically in a state of perfect order, $q=1$; while for $T \geq T_0$, there is no long-range order, and $q=0$. The order parameter q therefore be-

haves rather like the molecular field of the familiar Curie-Weiss theory. The authors have, in fact, worked out these ideas at a similar level of accuracy to that of the Curie-Weiss theory, and they suspect that their conclusions must be inaccurate in similar ways, notably in a wrong prediction of the detailed shape of the cusp in $\chi(T)$: they are generously leaving the application of quantum mechanical refinements to other workers.

Their main conclusion, however, is the important one: that, even though the alloy may be neither ferromagnetic nor antiferromagnetic, it will nevertheless undergo a magnetic ordering transition which should manifest itself as sharp peaks in properties such as the magnetic susceptibility, and at a temperature which can in principle be predicted. This is a remarkable theoretical result, but one which is in excellent accord with reality as revealed by the experiments.

Mutating and mapping SV40

from Lois K. Miller

NUCLEIC acid biochemists are now able to manipulate the DNA genome of small animal tumour viruses with remarkable skill as illustrated by two papers from Paul Berg's laboratory (Shenk *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, 72, 989; 1975; and Carbon *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, 72, 1392; 1975). These workers have exploited the known specificities of several types of DNA nuclease to produce viral deletion mutants and map those mutants with astounding ease, accuracy, and rapidity.

Mutations in small animal viruses are difficult to map by the more conventional genetic methods because of their size, low recombination frequencies, and lack of different mutant types for three-factor genetic crosses. A technique, originally developed for Φ X174 (Weisbeek *et al.*, *Biochim. Biophys. Acta*, 224, 328; 1970; Hutchison and Edgell, *J. Virol.*, 8, 181; 1971), was modified by Lai and Nathans (*Virology*, 60, 466; 1974), and utilised to map temperature sensitive (ts) mutations of SV40. The technique relies on the ability of wild-type DNA fragments to rescue a mutant DNA genome when the two are properly annealed together and introduced to an appropriate cell. When wild-type restriction endonuclease fragments are employed, the mutation can be localised within a single fragment and the relative position within the genome is known since restriction nuclease fragments can be ordered by independent means. Al-

though it is known from the Φ X174 studies that fragments 30–50 nucleotides long can rescue mutant genomes, the achievement of such accuracy in mapping with this technique would require a tour de force on the part of the investigator. Nevertheless, marker rescue is a rapid direct approach to mapping ts mutations within a region of the genome 300–500 nucleotides in length.

Shenk *et al.* have developed a purely biochemical mapping technique which is based on the ability of S_1 nuclease, an endonuclease from *Aspergillus oryzae* which recognises regions of single-stranded DNA, to produce double-stranded cleavages at the mismatched region of a wild type–mutant heteroduplex. The technique is ideal for mapping small deletion mutations (too small to map by electron microscopy heteroduplex techniques) due to its rapidity and accuracy. Although Shenk *et al.* have also mapped ts mutations using this technique, it is apparent from their results that single-base mutations are not easily recognised by the S_1 activity. In mapping ts mutations, Shenk *et al.* observe very high backgrounds due to non-specific cleavage of the DNA and the 'nibbling' of the breathing ends of the double-stranded DNA by the S_1 nuclease. It would be interesting to scan the presently known repair endonucleases for an enzyme with higher specificity for single-base mismatched regions and couple such an enzyme with the S_1 nuclease technique.

There are several advantages the S_1 mapping technique has over the marker rescue technique. The S_1 technique, according to Shenk *et al.*, is accurate to 1% of the SV40 genome or approximately 55 nucleotides and thus gives ten times more accuracy with approximately equal ease. Since the S_1 technique is purely biochemical, it does not rely on infectivity and is potentially useful for mapping non-viral DNA (including eukaryotic DNA) or viral DNAs having low plaque forming units/ μ g DNA ratios (for example adenovirus 2). But since it is purely biochemical, caution must be taken that a second-site, silent mutation is not mistaken as the true mutation.

Carbon *et al.* have developed an excellent technique for producing deletion mutants of SV40. By digesting 25 to 30 nucleotides from the ends of a linear double-stranded DNA with the 5'-end specific lambda exonuclease, they produce shortened DNAs which, when introduced into monkey cells, yield circular deleted genomes in a manner similar to that previously reported by Lai and Nathans (*J. molec. Biol.*, **89**, 179; 1974) at a high frequency. By using appropriate combinations of DNA nucleases, it is

apparent that Carbon *et al.* will be able to produce deletion mutants not only at restriction nuclease sites but also at essentially random sites throughout the SV40 genome. Since these deletion mutations can be mapped readily by the procedure of Shenk *et al.*, such mutants should be most helpful in defining essential regions of the genome and the limits of the various genes.

Previous reports on isolating deletion mutants (Mertz and Berg, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4879; 1974); Lai and Nathans, *J. molec. Biol.*) have shown that certain deletion mutants are viable whereas other mutants are non-viable and must be carried with helper virus. The latter case presents problems in obtaining pure stocks of deletion mutants. It would be useful in this respect to develop stocks of helper virus which could contribute functions lacking in the non-viable deletion mutants yet be easily separable from the deletion mutant itself. It would also be useful to develop *in vitro* techniques for the production of specific conditional lethal mutants (such as ts mutants) at high frequencies.

Lipid on the run

from A. G. Lee

THE nightmare of anyone using probe molecules to study membranes is of awakening to find that all that has in fact been studied is the probe. Hence the tendency to seek confirmation of previous results using new probes and new physical techniques, on the principle that a nightmare shared is a nightmare halved.

The latest example of this concerns the measurement of the diffusion rates of lipids within the plane of a lipid bilayer. These measurements have generally been based on studies of the magnetic interactions between molecules in the membrane. If a spin-labelled steroid or lipid is incorporated into the membrane at high concentrations, then magnetic interactions occur between the unpaired electrons of the spin-labelled molecules, and this interaction is reflected in the shape of the electron spin resonance spectrum. If the spin labels are moving in the plane of the membrane, then the movement will modulate the magnetic interactions, the exact effect of the motion on the shape of the spectrum depending on the rate of the motion. In this way, Träuble and Sackmann were able to estimate a rate of lateral diffusion for spin-labelled androstan of about $1\text{--}2 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ (*J. Am. Chem. Soc.*, **94**, 4499; 1972). Devaux and McConnell

(*J. Am. Chem. Soc.*, **94**, 4475; 1972) obtained similar results, also with spin-labelled molecules, but using a kinetic technique to detect diffusion. A diffusion rate of this magnitude is of considerable interest since if the lipids in a real biological membrane were moving as fast, it would mean that a lipid could move from one end of a $1 \mu\text{m}$ long bacterium to the other in the order of a second.

An alternative approach was to look at the magnetic interactions between the protons of the lipid fatty acid chains which are reflected in the proton nuclear magnetic resonance line shapes. This has the advantage that it is possible to use natural phospholipids, with no perturbing spin-label, but it has the disadvantage that it is rather difficult to separate contributions to the linewidth from the diffusive motion and from other sources. However, the self-diffusion coefficients obtained in this way were identical to those obtained by electron spin resonance (Lee *et al.*, *Biochemistry*, **12**, 1650; 1973).

In the latest paper (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 1451; 1975) on lateral diffusion Brulet and McConnell have extended the theory of time-dependent magnetic interactions to the case of two-dimensional diffusion. This has enabled them to look in detail at the effect that a spin-labelled molecule incorporated into a lipid bilayer will have on the relaxation times of ^{13}C or ^1H nuclei of the lipids of the bilayer. Unfortunately, for the calculations to be at all tractable it was necessary to assume that the two-dimensional lateral diffusion of the spin label was confined to one single plane, at some fixed depth within the lipid bilayer. Similarly it had to be assumed that the nucleus of interest was also forced to diffuse laterally within a fixed plane. Neither of these assumptions is strictly valid, since it is known that phospholipids undergo extensive vibrational motion, so that positions in a direction perpendicular to the bilayer surface are constantly changing. Although it is not known what effect this will have on the results, it is more than a little comforting that what emerges at the end of the calculations is once again the magic number obtained by the other techniques— $2 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$.

It now seems very unlikely that these results for lipids in simple lipid bilayers will be overturned. When we turn to the intact biological membrane, however, an interesting problem emerges. If all the lipids in biological membranes were to have such high diffusion rates, then there could be no persistent interactions between specific proteins and lipids in the membrane, and the environment of the membrane proteins would be a weighted average of that provided by the heterogeneous mixture

Superconducting regions in lysozyme

from a Correspondent

GREAT interest was aroused at the Dielectrics Society annual meeting by Herbert Fröhlich's report of some experiments which show that an aqueous solution of the enzyme lysozyme contains regions which are superconducting. The report (now published: Ahmed, Calderwood, Fröhlich and Smith, *Phys. Lett.*, **53A**, 129; 1975) can be summarised thus.

Magnetic fields of order 600 Gauss were found to bring about very large changes in the dielectric constant of aqueous lysozyme solutions. Since for this to happen the magnetic field must be able to overcome ionic thermal fluctuations, the magnetic energy must act over a volume of the order of a million lysozyme molecules, which indicates the existence of a cooperative phenomenon capable of increasing the magnetic susceptibility in a

magnetic field.

This measurement was therefore made, and a 1% increment in bulk susceptibility was found on application of the 600 Gauss field. This is ten thousand times larger than the effect for an ordinary diamagnetic material and its disappearance about 800 Gauss indicates that it represents a Meissner effect in superconduction. Complicated temperature and concentration effects were found, including temperature hysteresis. It was postulated that in solution the ions form a layer structure, giving rise to an a.c. Josephson effect, that is, to the electric vibrations in biomolecules recently proposed by Fröhlich and reported in the Soviet literature. Physiological effects of magnetic fields would seem to be given physical respectability if a Meissner effect exists in a biomolecular solution.

of chemically distinct lipids present in that membrane. That goes against the considerable weight of experimental evidence suggesting specific interactions between particular proteins and lipid classes, with at least a ring of lipid bound relatively tightly to the protein in a long-lived interaction. So it seems that whereas the bulk of the lipid could be diffusing as fast as lipid in simple lipid bilayers, there might also be some lipid in contact with protein and exchanging only slowly with the bulk lipid.

In a recent paper, Warren *et al.* (*Nature*, **255**, 684; 1975) explore this possibility of relatively long-lived lipid-protein interactions with their simplified lipid-protein system, containing the (Ca^{2+} - Mg^{2+}) ATPase from sarcoplasmic reticulum. They have found that the activity of the protein is maintained as long as there are 30 or more phospholipid molecules for each protein molecule: with a smaller proportion of phospholipid there is an irreversible loss of activity. It has been suggested therefore that these 30 lipid molecules form a single shell of phospholipid bilayer around the protein, protecting it from denaturation. Further experiments have then shown that cholesterol, which on its own leads to a reversible loss of activity, cannot normally penetrate this lipid annulus. If, as seems likely, this proves to be a general property of membrane proteins, then the organisation of lipids within the membrane will be by no mean homogeneous. Each protein will be surrounded by a lipid microenvironment, the structure of the

protein and the nature of the micro-environment together determining the functioning of the protein. Only the bulk lipid will be free to diffuse within the membrane, and around each protein there will be an immobilised lipid annulus, whose composition will be determined by the structure of the protein. Even in the transitory world of membranology, not everything is in a state of flux.

Mammalian development, with chicks and newts

from Gillian M. Morris

The Ciba Foundation held a symposium on "Embryogenesis in Mammals" on June 16-19. The proceedings will be published by Associated Scientific Publishers.

THE symposium was held just ten years after the symposium on preimplantation states of pregnancy—ten years which have seen a tremendous creative surge in the field of vertebrate experimental embryology. With congenital anomalies an increasing medical problem, much of this creative energy has been applied to studying normal and abnormal postimplantation mammalian development. Whole embryo and organ culture techniques have contributed greatly to the accessibility and reliability of the systems under

investigation; however, in comparison with the work on other vertebrates, mammalian embryology appears to have embarked on an obstacle course beset with many frustrations. Thus it was fitting that two presentations on recent advances in avian and amphibian development were included in the symposium, and that four additional non-mammalian embryologists were present as discussants.

Death of a germ layer

More than one established dogma was challenged by the participants. The first was the demonstration by R. L. Gardner (University of Oxford) that the early differentiation of inner cell mass into 'endoderm' (hypoblast) and 'ectoderm' (epiblast) does not signify the establishment of cell lineages loyal to the historical concept of germ layers. The most important determinative factor in the differentiation of preimplantation and implanting embryos is cell position; thus chimaeras can be constructed whose primary endoderm-derived cells are detectably different from the rest of the embryo. Examination of such embryos at 15½ days showed that the whole embryo is derived from the so-called ectoderm. Other experiments demonstrated that even the germ cells are ectodermal in origin.

N. Skreb (Faculty of Medicine, Zagreb), describing a study carried out in collaboration with B. Levak-Svajger and A. Svajger (see *J. Embryol. exp. Morph.*, **32**, 445-467; 1975), showed that, when transplanted to a site under the adult kidney capsule, embryonic ectoderm retains the capacity to differentiate into all embryonic structures (including gut) up to the primitive streak stage, though not later. Endoderm alone was always resorbed, but this may simply be a further demonstration of the essential interactive role of adjacent mesenchyme and intercellular matrix in epithelial differentiation. Nicole Le Douarin (Université de Nantes), for instance, showed that grafts of portions of avian pharyngeal endoderm will not differentiate in the absence of mesoderm. The major part of her paper concerned that unique piece of ectoderm, the neural crest. Her elegant studies on quail-chick transfers are too well known to need description here (see, for instance, *Devl Biol.*, **41**, 162-184; 1975); as the chairman, Anne McLaren (University College, London) remarked, they are a source of envy to mammalian embryologists. A further characteristic of the primitive endoderm is its slow proliferation: M. Snow (University College, London) calculated that in the primitive streak stage mouse embryo, the cell cycle time in the endoderm is more than 15 h, compared to 6½ and

8 h in the other two layers.

The question of the developmental capacity of the primitive (or primary) endoderm is clearly as yet unresolved. But does the term 'germ layer' remain meaningful or useful? Small wonder that by the end of the symposium there was general agreement that the products of the first differentiation of the inner cell mass be designated 'epiblast' and 'hypoblast', in common with avian terminology.

A day of consideration of genetic aspects began with the questions: "How early do genes begin to function in mouse embryos?" and "Is there a genetic programme for the timing of gene expression?" Using β -glucuronidase as a specific product of gene expression in two strains of mice whose rate of synthesis of the enzyme differs, V. M. Chapman (Roswell Park Memorial Institute, Buffalo) suggested that the gene involved is expressed as early as the two-cell stage.

Postimplantation developmental abnormalities were described in a variety of trisomies in mice by A. Gropp (Lubeck Medical School). Like human trisomies, these are all associated with abnormal facial morphogenesis, but the mechanisms are unknown.

Gene action in abnormal preimplantation development of mouse embryos homozygous for the yellow allele of the agouti locus was demonstrated by R. A. Pedersen (University of California, San Francisco) by means of timelapse cinemicrography of cultured embryos. The abnormalities culminating in preimplantation loss consisted of delayed or arrested cell division during cleavage stages, and failure of extension and adhesion of trophoblast cells to the culture dish at the implantation period. One may speculate that the lethal gene affects the synthesis of one of the proteins which are involved in both spindle formation and cell motility or adhesion.

Abnormalities of cell motility and cell-cell interaction are characteristics of abnormal gene expression in *T*-locus mutant mice. In an ultrastructural study of the lethal *t^o*-homozygote, Martha Spiegelman (Cornell University Medical College) showed that newly formed primary mesenchyme cells lack subsurface microfilaments, do not make organised cell contacts, and fail to migrate away from the primitive streak. In *T*-homozygotes the abnormality involves defective development of the basement lamina of the neuroepithelial cells, which are consequently able to make close associations with underlying notochord and somite cells. Thus alterations in the cell surface are of major importance in the changing interactions between cell and environment. In this context, M.

Eddin (Johns Hopkins University) examined three types of antigen, and changes in the concanavalin A binding pattern, as indicators of alterations in cell surface components. The results confirmed that new antigens are expressed during the course of differentiation, and that their quantity alters. Con A receptors come and go on mouse embryonic cells: they increase up to the blastocyst stage, but in the blastocyst itself they disappear from trophoblast cells and are localised exclusively in the inner cell mass.

W. J. Rutter (University of California, San Francisco) showed that actual cell contact may not be necessary for differentiation of the pancreas. The epithelium will develop its characteristic glandular structure when in contact with sepharose beads coated with a factor derived from mesenchyme. The cell surface receptors influence the synthetic abilities of the cell, under appropriate external stimulation. In the context of the cell-cell interactions, the cell surface must be taken to include the surface coat. This was clearly demonstrated by R. O. Kelley (University of New Mexico, Albuquerque), using the epithelial-mesenchymal interaction during human limb morphogenesis as a model system, and an impressive armoury of techniques. The mesenchymal cells have both receptor and catalytic components of the adenylyl cyclase system, and the receptivity of this system to exogenous stimulation is potentiated by diminishing or removing the cell surface glycosaminoglycans (GAGs). The fact that trypsin reduces the thickness of cell surface GAGs should be borne in mind by all experimenters using this enzyme.

Although the stability of the differentiated state is still acceptable as a general rule, studies on transdifferentiation (well established in connection with regeneration in amphibia) have now been extended to avian embryos. G. Eguchi (University of Kyoto) showed that in the case of both chick and newt, cloned pigmented retinal epithelial cells will give rise to a few lens cells. The time period is about 90 days in the chick compared with less than 40 in the newt, but the process can be speeded up, *in vivo* as well as *in vitro*, by the use of a small crystal of nitrosoguanidine (a potent mutagen/carcinogen). The outcome of similar experiments on mammalian cell lines is awaited with interest.

Those present at the symposium spanned a great many of the component areas of this exciting field of research. The discussions were both wide-ranging and controversial. Such a stimulating symposium can only add to the accelerating impetus which all areas of developmental biology are undergoing at the present time.

A comet in the Plough

from Robin Scagell



THE first comparatively bright comet to appear since the ill-starred Kohoutek is in the evening sky at the moment, and is making up for its predecessor's fickleness by being readily observable with binoculars. It could well provide an interesting sight within the next few weeks.

Comet Kobayashi-Berger-Milon, named after its three independent discoverers, was first spotted from Japan on July 2, and has the designation 1975h. It has been moving through the evening sky towards the Sun, to which it makes its closest approach on September 5. Much to the delight of those who spent cold and fruitless vigils trying to glimpse Comet Kohoutek, 1975h has passed almost through the zenith for observers in Britain and America and was easily seen even on nights of full Moon. By July 24, although no tail was noticeable, the object appeared as a fairly large circular diffuse patch of light of about fifth magnitude—just visible to the naked eye.

Kobayashi-Berger-Milon represents a good run of the mill comet which is worth following. It has even had the good sense to appear to mingle with the stars of that best known of all northern constellations, Ursa Major—the Plough or Big Dipper. At the beginning of August it will pass close to the well known double star Mizar, the middle star in the handle of the Plough or Dipper, and should be easily visible with even the smallest pair of binoculars.

Throughout August it will move steadily south-eastwards until it reaches perihelion on September 5 in Leo Minor, where it will best be seen in northerly latitudes at twilight. After perihelion it will become less readily visible.

articles

Three-dimensional structure, function and genetic control of immunoglobulins

Roberto J. Poljak*

Recent crystallographic analyses of the three-dimensional structure of immunoglobulins have shown how it is related to their binding affinities. Considered with evidence from sequencing, it is beginning to cast some light on how the genetics of immunoglobulins could have evolved to maximise binding diversity with a minimal genetic load.

SERUM antibodies are glycoproteins generically denominated immunoglobulins. Although in recent years much progress has been made in analysing experimentally induced antibodies¹, the structure of immunoglobulins has been most intensively studied using human and murine myeloma proteins which provide homogeneous material in the quantity required for structural analysis. It is now widely accepted that antibodies and myeloma immunoglobulins are closely related molecules. The antibody response against a specific antigen (or hapten) is usually degenerate, consisting of a heterogeneous population of molecules, a fact which complicates structural analyses. In contrast, myeloma immunoglobulins are homogeneous but the elucidation of their hapten binding specificity may require an intensive search.

Several classes (or isotypes) of immunoglobulins (Ig) are present in normal or pathological sera and of these the IgG (or γ globulin) class is the most abundant and has been most intensively studied. A diagrammatic representation of a human IgG immunoglobulin is shown in Fig. 1. The comparison of the amino acid sequences of internal segments of the heavy (H) and light (L) polypeptide chains have led to the recognition of homology regions about 110 amino acid residues long². The homology region designated as C_L (Fig. 1) is constant in amino acid sequence and characteristic of a given subclass (κ or λ) of L chains which are present in all immunoglobulin classes. C_H 1, 2, 3 are also constant in sequence and characteristic of a given class or isotype of immunoglobulin defined by its H chain. When the amino acid sequences of different immunoglobulins are compared, the regions designated as V_L, V_H (Fig. 1) show variability, in particular around positions 25–30, 50 and 95–100 in V_L and 30, 55–60 and 105–110 in V_H, positions which are called "hypervariable"³. These hypervariable regions have been postulated by many workers to be the determinants of the conformation and specificity ("complementarity") of an antibody combining site, and to be closely related to the "idiotypic"^{4,5} antigenic markers which are unique to immunoglobulins secreted by a clone of cells.

Three-dimensional structure of immunoglobulins

Models of Fab' New obtained by X-ray crystallographic analyses at 6 Å, (ref. 6), 2.8 Å (ref. 7) and 2.0 Å (ref. 8) resolution showed that Fab' consists of two globular domains, V and C, separated by an internal space accessible to solvent. Each domain consists of two globular subunits corresponding to the V_L and V_H homology regions in the V domain, and to C_L and C_H1 in the C domain. V_L and C_L as well as V_H and C_H1 are

joined by narrow, linear regions by which the L and H polypeptide chains cross from the V to the C domain. These regions are exposed, accessible to solvent and to attack by agents such as proteolytic enzymes. The overall structure is that of four "homology subunits" in tetrahedral arrangement. In agreement with their sequence homology and the ensuing postulate that they arose from a common ancestral gene², V_L, V_H, C_L and C_H1 share a basic pattern of polypeptide chain folding although V_L and V_H include an additional loop of polypeptide chain (Fig. 2). In each homology subunit two irregular, roughly parallel β sheets consisting of strands of antiparallel polypeptide chain surround a tightly packed interior of hydrophobic side chains including the intrachain disulphide bond. In the variable homology subunits V_L and V_H, the hydrophobic interior consists of invariant or semi-invariant amino acid side chains. The same overall pattern of polypeptide chain folding was observed in the 3.5-Å resolution structure of the Mcg L-chain dimer⁹. Different arrangements of interchain disulphide bonds which occur in other immunoglobulin molecules could be explained on the basis of the Fab' New model. Since these different disulphide bonds occur in macroglobulins (IgM), in

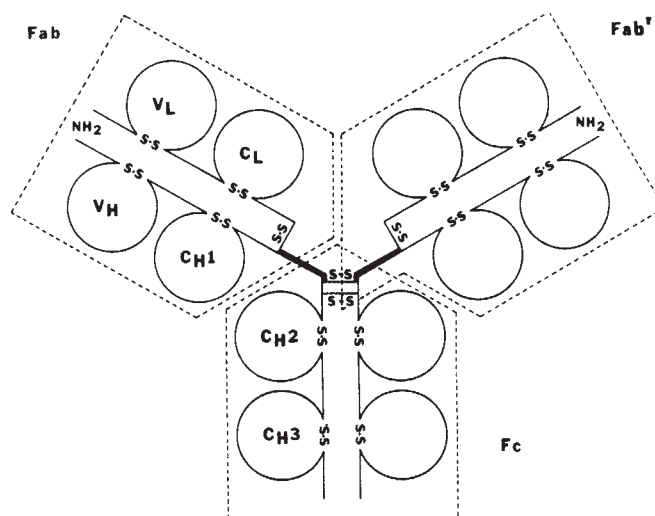


Fig. 1 Diagrammatic structure of a human IgG1 molecule. The light chains are divided into two homology regions, V_L and C_L. The thicker lines in the heavy chains correspond to the "hinge" region. The four homology regions (V_H, C_H1, C_H2, and C_H3) of the heavy chains, the interchain and intrachain disulphide bonds, the N-terminal region of both chains, and the major fragments (Fab, Fab', Fc) are indicated. Reproduced from Poljak³.

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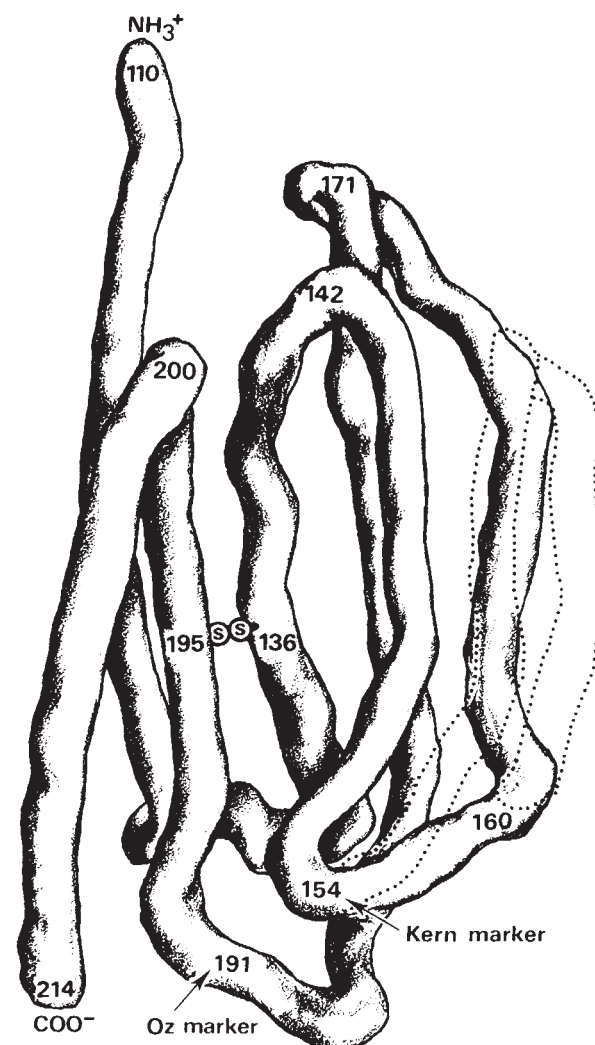
secretory immunoglobulins (IgA) and in other immunoglobulin isotypes, it was postulated⁷ that the basic immunoglobulin fold (Fig. 2) is present in all classes of immunoglobulins. This postulate was given substantial support by the results of the crystallographic analysis¹⁰ of the Fab fragment from the McPC 603 IgA, a murine immunoglobulin secreted by cells of an experimentally induced mouse myeloma tumour. The H (α) and L (κ) polypeptide chains present in this immunoglobulin are of different isotype from the corresponding chains of the human Fab' but the overall three-dimensional structure is similar. Further, the high resolution structure of the variable part of a human κ chain (a V κ dimer) indicated the same folding of the polypeptide chain¹¹. At the present stage of the structural analysis of immunoglobulins the postulate that they all share a common pattern of three-dimensional structure (Fig. 2) can be accepted with reasonable confidence. The "hinge" region connecting Fab to Fc is not included in this pattern; different isotypes of immunoglobulins differ in this region which determines a segmental flexibility¹². Within one isotype of immunoglobulin, the region that determines the antigen combining site (or active centre) makes for variability in structure and function.

The combining site

The atomic resolution model of the human Fab' fragment⁷ showed that the hypervariable regions of both L and H chains occur at one end of the molecule, in spatial proximity and fully exposed to solvent with all the characteristics of a combining site. A similar observation was made in the structure of Fab from the McPC 603 IgA (ref. 10). Thus, different immunoglobulins will present a unique, idiotypic structure at this end of the molecule. In the L chain dimers Mcg⁹ and Re¹¹ there is a cavity surrounded by the hypervariable regions of both chains which resembles the combining site of the Fab fragments, although it is wider and symmetrical around a central twofold rotation axis. The combining site of IgG New consists of a shallow groove, about 15×6 Å, with a depth of about 6 Å. The active centre of McPC 603 is deeper (about 12 Å) than that observed in IgG New. This increased depth can be attributed in part to the fact that the first hypervariable region of the κ (L) chain of McPC 603 is six amino acids longer (by an "insertion" of the type shown in Fig. 3 for human V κ_{HII} sequences) than other murine and human κ and λ chains. This inserted sequence protrudes to form a "deeper" active site. It follows that the pattern of insertions and/or deletions, characteristic of the hypervariable regions (in particular around residue 105 in human H chains) will be the major determinant of the dimensions of the active site. Variations in sequence or amino acid replacements which are also a characteristic of those regions will determine the chemical environment of the site. Thus, different antibodies may display unique antigen complementarity sites although they share a common three-dimensional structure. A vast number of recognition specificities can be incorporated in this design, although a constant, compact immunoglobulin fold is maintained. A further consideration is that, since both H and L chains contribute to the combining site, random pairing could give rise to a large number of different combining sites (n^2) with a much smaller number (n) of H and L chains, further extending the economy of structural design. The number of germ line structural genes required to encode all necessary H and L chains in an immunologically mature animal would thus approach a reasonably small value ($n \leq 10^3$) when compared with the total number of structural genes in the genome of vertebrates. Evidence of recombination of H and L chains *in vitro* has been obtained in several laboratories (for reviews see refs 13 and 14); and the possibility that recombination is restricted to mutually compatible H and L chains has also been submitted to limited experimental tests^{13,15}. Experimental tests of *in vitro* recombination of H and L chains would however have to cover all possible subgroup and isotype permutations (subgroup is used to designate a set of V_H or V_L sequences which are similar or more closely related to each

other than to those of another subgroup). A study of this problem by analysis of the interactions between V_L and V_H in the three-dimensional structure¹⁶ reveals close interactions between amino acid side chains at positions 35, 37, 42, 43, 86 and 99 in V_L and at positions 37, 39, 43, 45, 47, 95 and 108 in V_H. These positions are occupied by constant residues or by fairly conservative replacements in human V_L and V_H sequences as well as in V_L and V_H sequences that have been studied from other animal species. Furthermore the amino acid sequences of human κ and λ chains are very similar at these positions: almost all human L chains have Tyr (35), Gln (37), Ala (42), Pro (43), Tyr (86) and Phe (99). L chain positions 88 and 90 occur at the active site and also seem to provide for V_L-V_H interactions but the importance of these interactions is more difficult to assess because they could be affected to some degree by the conformation of the last hypervariable region of V_H; even so there are preferred residues at these positions: Gln (88) and Trp/Tyr (90). In the C_L region κ and λ chains share the sequence of residues which interact with C_{H1}. Thus, the interactions between V_H and V_L, which should provide the stereochemical basis for their association, seem to be independent of H and L chain subgroups or even L chain class. Although preferred associations could occur under genetic control there is no apparent structural basis for restricted pairing. Different H, L chain associations could be expressed under selective stimulation by antigens, thus making use of all the structural information contained in the genome.

Fig. 2 Diagram of the basic 'immunoglobulin fold'. Solid trace shows the folding of the polypeptide chain in the constant subunits (C_L and C_{H1}). Numbers designate L (λ)-chain residues, beginning at "NH₃⁺" which corresponds to residue 110 for the L chain. Broken lines indicate the additional loop of polypeptide chain characteristic of the V_L and V_H subunits.



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III	M11	Asp	Ile	Val	Leu	Thr	Gln	Ser	Pro	Leu	Ser	Leu	Pro	Val	Thr	Pro	Gly	Glu	Pro	Ala	Ser	Ile	Ser	Cys	Arg	Arg	Ser	Ser	Gln	Asn	Leu	Glu	Glx	Ser	Asx	Gly	Asx	--	--	--	--	Tyr	
	Tw	Asp	Ile	Val	Met	Thr	Gln	Ser	Pro	Leu	Ser	Leu	Pro	Val	Thr	Pro	Gly	Glu	Pro	Ala	Ser	Ile	Ser	Cys	Arg	Ser	Ser	Gln	Ser	Leu	Ser	Glx	Ser	Ser	--	--	--	--	--	--	Pho		
	Cus	Glu	Ile	Val	Leu	Thr	Gln	Ser	Pro	Leu	Ser	Leu	Pro	Val	Thr	Pro	Gly	Glu	Pro	Ala	Ser	Ile	Ser	Cys	Arg	Arg	Ser	Ser	Gln	Ser	Leu	Leu	Asp	Ser	Gly	Asx	Gly	Asn	Thr	--	--	Tyr	
Bates	Asp	Ile	Val	Met	Thr	Gln	Ser	Pro	Leu	Ser	Leu	Pro	Val	Thr	Pro	Gly	Glu	Pro	Ala	Ser	Ile	Ser	Cys	Arg	Arg	Ser	Ser	Gln	Ser	Leu	Leu	His	Ser	Asx	Gly	Asx	Gly	Asn	Thr	--	--	Tyr	

In human L chains, subgroups can be defined by (or correlated with) the presence of insertions (or deletions) in the first hypervariable region (V κ , V λ) and also in the third hypervariable region (V λ). Consequently, these subgroups define different conformations of the active site, as discussed above. For example, human κ chains of subgroup III (V κ_{III} , see Fig. 3) will delineate a "deeper" active site such as that observed in the murine myeloma protein McPC 603 (ref. 10) than will a κI or a λ chain. Since murine λ chains seem to lack the pattern of deletions and/or insertions at the hypervariable regions characteristic of murine κ and human κ and λ chains, they should be expected to make a smaller contribution to the diversity of antibodies. Thus the number of genes encoding λ chains or their expression in mice would be limited relative to κ chains as reflected in the κ/λ serum ratio¹⁷.

An increasing number of human and murine myeloma proteins has been shown to bind ligands in a reaction that closely resembles that of a specific hapten-antibody system^{18,19}. A number of mice myeloma proteins that bind phosphorylcholine share idiotypic determinants with induced anti-phosphorylcholine antibodies indicating the antibody function of these immunoglobulins¹⁸. Although McPC 603 IgA does not share this idiotypic specificity it binds phosphorylcholine. Crystallographic analysis of the Fab-phosphorylcholine complex revealed that phosphorylcholine binds close to heavy chain residues, occupying a relatively small region of the combining site. No major conformational change was observed in the Fab structure after ligand binding. IgG New binds a number of ligands such as menadione, orceine, uridine and others with a low affinity constant ($K_0 = 1 \times 10^3$ L/M), and a γ -hydroxy derivative of vitamin K₁ (vitamin K₁OH) with a higher affinity constant ($K_0 = 1.7 \times 10^5$ L/M)²⁰. Crystallographic studies²¹ of the complexes between these ligands and Fab' New showed that vitamin K₁OH binds at the combining site, making contact with a number of amino acid chains of the hypervariable regions of the L and H chains; the other ligands bind at the same site occupied by the naphthoquinone moiety in the vitamin K₁OH-Fab' New complex. Since the affinity constants for these compounds are lower than that observed for vitamin K₁OH it was concluded that the total binding energy in the Fab' New-vitamin K₁OH complex is derived from contacts made by the naphthoquinone moiety and by the phytyl chain with the side chains at the active site. As in the case of the McPC 603 Fab, no major conformational change took place after binding of vitamin K₁OH to Fab' New. These studies provided firm experimental evidence for the concept that the hypervariable regions of H and L chains determine the conformation of the combining site and a preliminary structural model for the interactions between antigens and antibodies. Ligand binding studies have also been conducted with the Mcg L-chain dimer

Using the amino acid sequence and the three-dimensional structure of Fab' New as a basic structural model, a correlation between the structure and function of the well characterised MOPC 315 anti-2,4-dinitrophenyl (DNP) mouse myeloma protein has been made⁸. The L (λ) chains of IgG New and MOPC 315 IgA are highly homologous in their first and third hypervariable regions where they contain a similar number of amino acid residues; also, the sequences of V_H New and V_HMOPC 315 are closely related in number of amino acid residues at their hypervariable regions. Therefore, the V_L and V_H sequences of MOPC 315 can be made to fit the basic structure of the active site of IgG New. In this tentative model, MOPC 315 IgA has a shallow combining site with a high density of Trp, Tyr and Phe residues in close correlation with the observed specificity of MOPC 315 IgA for DNP, menadione, flavin mononucleotide, vitamin K₁ and other haptens which include aromatic rings in their structure²³. IgG New and IgA MOPC 315 have nearly equal avidities for vitamin K₁ and each binds additional ligands not bound by the other. This observation of two binding sites that partially overlap in their ligand-binding spectrum can be taken as support for the postulate that antibodies are multispecific^{24,25}. Thus, different antibodies will cover a wide spectrum of antigens with partially overlapping domains of specificity, leading to degenerate immune responses. The mode of binding could be common to a group of chemically related ligands such as DNP, vitamin K₁, menadione, and so on, which share aromatic ring structures. Different affinity constants will be observed in these ligand-antibody complexes reflecting a different number of additional chemical contacts and interactions. A gene pool encoding these multispecific antibodies would be smaller than one encoding monospecific antibodies and each of its structural genes would naturally be subject to repeated induction and expression. The selective advantages of carrying such a genetic pool would ensure its maintenance against genetic drift and spontaneous deleterious mutations.

Variations in the amino acid sequences of the L and H chains can be analysed in terms of their three-dimensional structure. A striking example of the relationship between a deletion (or insertion) and the tertiary structure is observed at positions 27a, 27b and 27c in human L (λ) chains. These residues form part of a single turn helical loop which can be deleted without creating a gap or other major alteration in the path of the polypeptide chain⁷. Deletions also occur in segments of the polypeptide chains of immunoglobulins outside the hypervariable regions. Two examples with a clear structural correlation can be given

here. The first is that of the deletion of residues numbered 54 to 60 in the amino acid sequence of the L chain of IgG New⁷. These residues very nearly constitute the loop of polypeptide chain which is characteristic of the variable regions (V_L , V_H) of immunoglobulins and which does not occur in the constant regions C_L , C_H1 (Fig. 1). An integral deletion of this loop can be easily visualised in a three dimensional model of V_L (Fig. 2) and evidently results in a stable tertiary structure similar to that of C_L . A second example is that of the deletion of an entire homology region, which occurs in H chains synthesised by human tumour cells in the "heavy chain disease"²⁶ and in murine myeloma H chains^{27,28}, beginning at the start of the C_H1 region (position 119) or before, and ending at position 215, close to the beginning of the C_H2 region. In the three dimensional structure the sequence . . . Val-Ser-Ser shared by γ and μ chains²⁹, clearly constitutes the end of V_H , and after a sharp bend, position 119 marks the beginning of the C_H1 homology subunit. Complete deletion of C_H1 should not affect the molecular interactions of C_H2 and C_H3 , leading to a compact, stable Fc-like molecule with an N-terminal appendix of variable length.

Variations in the amino acid sequences of V_L and V_H which do not affect the length of the polypeptide chains ("point mutations") also have structural correlations⁸. For example, invariable or semi-invariable glycine residues occur at bends (hairpin loops) of the polypeptide chains or at internal points of close contact between two strands of polypeptide chain where larger side chains could not be accommodated; other constant or nearly constant residues are involved in close contacts between V_H and V_L (discussed above), make intrachain hydrogen bonds, or occur in the tightly packed interior of the structure between two β sheets. Multiple mutations that alter these residues to other sequences compatible with an energetically stable, constant three-dimensional structure can best be explained by a process of evolutionary germ-line gene divergence rather than by selection of random somatic mutations. In contrast, the amino acid residues that constitute the hypervariable regions and the antigen complementarity site are not subject to visible structural constraints.

A comparison of the amino acid sequence of β_2 -microglobulin with those of the constant regions of immunoglobulins reveals a striking homology³⁰ indicating that the overall polypeptide chain folding of β_2 -microglobulin is similar to the folding of the C regions of immunoglobulins (Fig. 2). This homology includes the hydrophobic amino acid side chains by which C_L and C_H1 interact to form a compact C_1 structural domain. The extent of sequence homologies with the C regions of immunoglobulins further suggests that β_2 -microglobulin interacts with another polypeptide chain that shares the basic immunoglobulin fold (Fig. 2). This common structural folding may be reflected in the amino acid sequences of the proteins bearing the murine H-2K, H-2D or the human HLA-K, HLA-D antigenic specificities and which interact with β_2 -microglobulin in cell membranes. The structural similarities

between histocompatibility antigens and immunoglobulins suggest a common genetic origin and subsequent steps of translocation and duplication³¹.

The basic polypeptide chain folding of immunoglobulins (Fig. 2) has recently been observed in the Cu, Zn enzyme superoxide dismutase from bovine erythrocytes³². A comparison of the structure of superoxide dismutase with that of the C and V regions of immunoglobulins indicates that the basic framework pattern of β sheets is present in all three; in superoxide dismutase and in the V regions there are insertions (such as in the hypervariable regions) which presumably have been positively selected through evolutionary events to optimally fulfil a specific biological function. Since the superoxide dismutase activity is present in many organisms including prokaryotes the "immunoglobulin-fold" may indeed turn out to be the expression of a widespread, ancient gene.

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Signal-to-noise ratio of electron micrographs obtained by cross correlation

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The signal-to-noise ratio of electron micrographs can be determined by two-dimensional digital cross correlation even though neither signal nor noise can be analysed separately. Such measurements suggest how best to make use of the electron microscope.

High resolution electron micrographs contain a large amount of detail that is not related to the object structure and is commonly referred to as noise. The relative amount of significant information compared with noise in the image can be expressed in terms of the ratio of signal variance to noise variance, or signal-to-noise (s/n) ratio. The s/n ratio is an important criterion for

the quality of the electron micrograph, and for the imaging process by which it is obtained. Unfortunately, no exactly defined electron microscopic model object is available from which the relative portion of the signal variance in the image could be inferred; hitherto, therefore, the s/n ratio has been a merely theoretical concept. Using a cross-correlation technique, we have studied the defocus dependence of the s/n ratio for a carbon film micrograph and found a characteristic behaviour which closely follows the predictions of linear contrast transfer theory, if one takes into account the effects of partial coherence and energy spread and assumes purely elastic scattering.

Our results support the view that, for thin biological objects and bright field illumination, elastic scattering plays the dominant role in contrast formation. They also stress the importance of the underfocus range for practical work and suggest a way of achieving accurate reproducible focusing of instrument with on-line image readout.

The technique

It has been suggested¹ that the sample cross-correlation coefficient could be used for estimating the s/n ratio of band-limited stochastic time functions, if two different versions of the desired signal disturbed by uncorrelated noise are available, each as a series of N time samples taken at a rate equal to twice the bandwidth. The sample cross-correlation coefficient of two series x_i, y_i with zero lag is defined as

$$r_N = \frac{\langle (x_i - \langle x_i \rangle)(y_i - \langle y_i \rangle) \rangle}{\{ \langle (x_i - \langle x_i \rangle)^2 \rangle \langle (y_i - \langle y_i \rangle)^2 \rangle \}^{1/2}}$$

where the angle brackets denote averaging. Assuming stationary Gaussian zero-mean statistics for both signal and noise one obtains the s/n estimate

$$\alpha = \exp[-2/(N-3)](r_N/(1-r_N) + \frac{1}{2}) - \frac{1}{2} \approx r_N/(1-r_N) \quad \text{for large } N \quad (1)$$

The formula for large N can be shown to be identical with the formula that generally relates the s/n ratio of two continuous series to their cross-correlation coefficient, irrespective of the statistical distribution of noise and signal. In image processing application where N is of the order of 10,000, the Gaussian assumption can therefore be dropped.

We have adopted this method of s/n measurement for the quantitative analysis of electron micrographs of a thin amorphous carbon film, and found excellent agreement between the defocus dependence of α obtained from two-dimensional digital correlation, and the theoretical behaviour expected for weakly elastically scattering objects ('weak phase objects').

In these conditions, the action of the electron microscope can be considered² as that of a linear system with transfer function $H(k)$ (k spatial frequency), with the projected potential of the object as 'input' and the image intensity recorded on the photographic plate as 'output'. The transfer function³ describes the distorting and resolution limiting effects due to spherical aberration, defocusing, axial astigmatism, energy spread⁴ and partial coherence^{5,6}. Assuming an input with power spectrum $P(k)$ and additive signal-independent noise on the output side, the s/n ratio of the output becomes proportional to the signal variance⁷.

$$\text{var}(s) = \int |H(k)|^2 P(k) dk \quad (2)$$

where the integration extends over the range of spatial frequencies within the bandlimit of the system.

Studies of carbon film

Pairs of electron micrographs of a thin (≈ 100 Å) carbon film were available, each pair taken with a different defocus but in otherwise identical conditions. Assuming a stable specimen, each pair contains the same signal portion distorted by the instrument in a characteristic way, with a superposed noise

portion that is due to the photographic grain and electron noise and is by first approximation additive to the signal. For the experiment, a JEM 100B electron microscope was used at 100 kV, $\times 200,000$ magnification and large objective aperture (≈ 0.5 Å⁻¹). Defocus values and astigmatic focus difference (250 Å) were determined by optical diffraction using Thon's⁸ method of evaluation, whereas the divergence of illumination (semiangle 2.7×10^{-4} rad) and the energy spread (1.8 eV) were obtained from bandlimit measurements (J.F., unpublished) using Young-type optical interference⁹ for each micrograph pair. From these parameters, the transfer function can be constructed for any defocus value (Fig. 1). As power spectrum, the intensity curve for elastic scattering of thin amorphous carbon films as obtained by electron diffraction¹⁰ was used. The overall behaviour of this function can be approximated in the interesting spatial frequency range by $P(k) \sim \exp(-|k|/\mathcal{H})$ with $\mathcal{H} = 0.18$ Å⁻¹. Since the highest bandlimit observed in the optical interference experiment was 0.38 Å⁻¹, the integration limit for the computation of the integral (2) was set at 0.4 Å⁻¹. For simplicity, the transfer functions were taken to be rotationally symmetric. As the astigmatism is very small, this approximation was expected to cause no appreciable error. The theoretical s/n ratio so obtained is shown in Fig. 2 as a function of defocus. For comparison, the case of a 'white' object spectrum $P(k) = \text{constant}$ was also considered (Fig. 3).

Broadly, the s/n ratio increases with increasing defocus and shows an oscillatory behaviour at positive defocus (that is, underfocus), with the maxima lying just above the defocus positions $[(2n+1)C_s\lambda]^{1/2}$; $n = 0, 1, 2, \dots$ (C_s spherical aberration constant, λ electron wavelength) where large transfer intervals appear in the transfer function. For carbon scattering, the absolute maximum is assumed at $n = 0$ ('Scherzer focus'), whereas it appears at higher defocus values for 'white' scattering. Even if one ignores the oscillations, the curve is remarkably asymmetric about $\Delta z = 0$. Computations with different illumination and energy spread parameters show that this behaviour is typical for imaging conditions where the resolution is limited by illumination divergence rather than energy spread.

The electron micrographs were copied from plates (Ilford EM4) on to fine grain film (Ilford N4E.50). This process is linear within 4% in the interesting density range, as checked by calibration, and does not affect the s/n ratio appreciably. The images were scanned using a Joyce-Loebl Scandig densitometer, with a sampling distance of 25 μm , corresponding to optimum sampling¹¹ of data with a bandlimit at 0.4 Å⁻¹. The digital data were recorded on magnetic tape and processed using the IMPROC image processing software system¹². The images were aligned using cross correlation¹³, and the orientation checked by rotational correlation techniques¹⁴. For the computation of the cross-correlation coefficients, matrices of size 128×128 were selected from the scanned data. Since a sizeable portion of the full cross-correlation matrix had to be examined in the context of another study, the computation was done by using the Fourier relation, by forming the conjugate product of the respective discrete transforms, inverse transformation, and appropriate normalisation. As a check on the assumption of stationarity, the cross-correlation coefficient was computed for different areas of the scanned image field, and was found

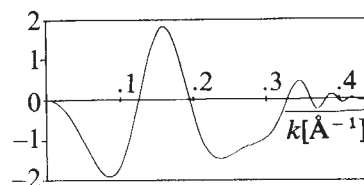


Fig. 1 Electron microscopic phase contrast transfer function for defocus $\Delta z = 1,860$ Å, spherical aberration constant $C_s = 1.8$ mm, electron wavelength 0.037 Å, chromatic aberration constant $C_c = 1.4$ mm, energy spread halfwidth $\Delta E = 1.8$ eV, and Gaussian source distribution with halfwidth 5.4×10^{-4} rad. The transfer function is computed from the coherent transfer function by using the envelope representations^{5,6}.

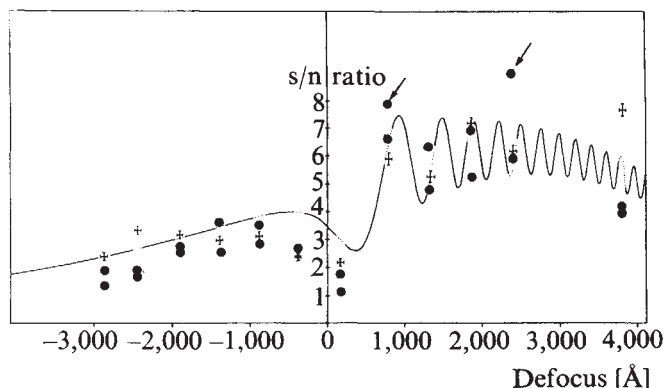


Fig. 2 Theoretical s/n ratio of electron micrographs of carbon as a function of defocus, and experimental s/n ratios α and $\tilde{\alpha}$ of a defocus series obtained from cross correlation (error bars) and from variance measurements (dots), respectively. The theoretical curve was scaled to fit the α measurements at $\Delta z = 790, 1,320, 1,860$ and $2,390$ Å optimally. The sections of the theoretical curve which lie within the horizontal error margins of the α measurements are marked by dotting.

reproducible within 5%. The s/n ratio α was calculated using formula (1). The result was corrected for the decrease of the s/n ratio due to the densitometer. Detailed analysis leads to a correction formula of the form

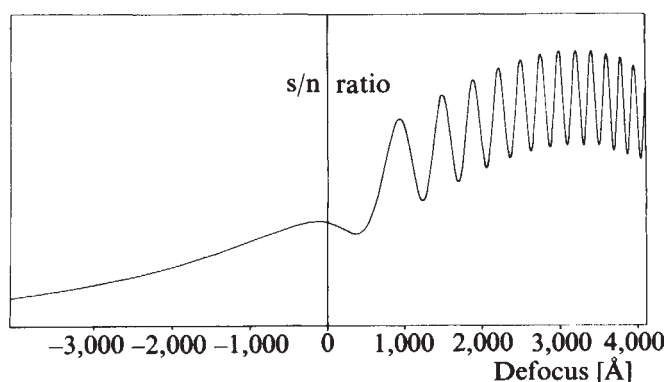
$$1 + 1/\alpha_{true} = (1 + 1/\alpha)/(1 + 1/\alpha_d)$$

where the s/n ratio of densitometer measurement, α_d , is obtained from the cross correlation coefficient of two scans of the same image. For our measurements, α_d was found to be between 50 and 90. The resulting α_{true} values are plotted in Fig. 2, with the horizontal bars indicating the inaccuracy of defocus determination, estimated at ± 50 Å, and the vertical bars the error limits due to the α_d range and the fluctuation of r when measured in different areas. The scaling factor for the theoretical curve was chosen in such a way that optimum fit occurs for the measurements at $\Delta z = 790, 1,320, 1,860$ and $2,390$ Å.

Interpretation

Theoretically, we expect some deviation of the measured values from the theoretical curve because the latter does not account for the change of noise variance with the signal variance that can be expected for a Poisson process. The defocus dependence of the experimental values is nevertheless in excellent agreement with the predicted behaviour: the measured s/n values are low and follow a flat curve at negative defocus, they then jump to a high value between the Gaussian and the Scherzer focus, and follow the predicted variations at the sampled defocus positions. (Corroborating the detailed structure of the theoretical curve at positive defocus by experimental data would be a formidable task with the method used, involving the evaluation of dozens

Fig. 3 Theoretical s/n ratios of electron micrographs for 'white' object scattering with arbitrary scale.



of micrograph pairs. It would be more adequate for this purpose to analyse electron micrographs obtained with a tilted stage which contain the entire defocus range of interest at once¹⁵. Data of this type are being evaluated at present, so that results on the continuous defocus dependence of the s/n ratio will soon be available (Kübler and Downing, private communication). As an independent check of the experimental values, we have computed the variances of all image pairs $\text{var}(i)$, and the variance of an image obtained with free electron exposure in the same optical density range, $\text{var}(n)$. From these variances we obtain again with the assumption of additive superposition and uncorrelated noise,

$$\tilde{\alpha} = (\text{var}(i) - \text{var}(n)) / \text{var}(n)$$

(Strictly speaking, these s/n estimates should be closer to the theoretical curve than the ones obtained by correlation, since they are obtained with the assumption of signal-independent noise variance.) We found good agreement between α and $\tilde{\alpha}$ (dots in Fig. 2) in the range of small α , and general agreement for large α . The two measurements marked by arrows belong to images whose histograms show a sharp peak on top of a Gaussian appearance. The origin of this artefact, which is missing in the corresponding twin image histograms, is unknown but it is thought that it is responsible for the large deviation of $\tilde{\alpha}$. On the other hand, no reason could be found for deviations at $\Delta z = 1,860$ Å and $3,900$ Å. These discrepancies will be investigated further.

Several conclusions can be drawn from the results of our study: (1) the satisfactory agreement between experimental and theoretical defocus dependence suggests that the linear transfer theory gives an adequate description of contrast formation in the conditions prevailing in the experiment, which are typical for electron microscopy of thin biological specimens; (2) in particular, since the fit of the theoretical curve to the experimental data was achieved by using a scaling factor only, it can be concluded that all those scattering processes ignored in our treatment, which could produce an image with defocus-dependent variance, make only a negligible contribution to the image contrast, such as inelastic scattering¹⁶; (3) with regard to efforts to retrieve the original object function by restoration techniques, it is important to know that the amount of information is maximised at distinct defocus values. For our experimental conditions, the first four maxima have approximately the same height. On the other hand, it is known from the study of partially coherent transfer functions¹⁷ that with increasing defocus the relative portion of high resolution information passed to the image increases. It may therefore be preferable, for the purpose of subsequent processing, to use defocus values larger than the Scherzer focus in the experiment, even though the electron micrographs may become less intelligible for direct interpretation; (4) as a practical conclusion, our measurements show that for the normal partially coherent illumination used, the gain in s/n ratio lies between two and three if one goes from overfocus to underfocus; (5) in the underfocus range, the positions of maxima and minima are fixed for a given instrument and may therefore be used as absolute focus references, possibly in connection with an objective current wobbler and on-line image readout, complementing methods recently proposed for controlled focusing¹⁸.

The method of measuring the s/n ratio by cross correlation is not restricted to phase contrast electron microscopy, or electron microscopy for that matter; it could be used quite generally, as a performance criterion of electron optical and light optical systems as well as new imaging and recording techniques.

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E. coli membrane lipid alteration affecting T4 capsid morphogenesis

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An Escherichia coli mutant, hd B3-1, cold sensitive for T4⁺ capsid assembly is described. The hd B3-1 character seems to be caused by altered lipid components of the bacterial inner membrane. The genetic locus for the hd B3-1 phenotype is designated fat A and is closely linked to proline.

THE *E. coli* B inner (cytoplasmic) membrane has been implicated in the assembly of bacteriophage T4⁺ heads. Electron micrographs of sections through cells fixed shortly after infection show amorphous lumps of viral head proteins associated with the bacterial inner membrane. These lumps form intermediate membrane-bound structures called τ particles which mature into empty phage heads that move from the inner membrane to the cytoplasm, where they are filled with DNA¹. Host factors thus seem to be involved in T4⁺ head morphogenesis, and we, as well as other investigators, have been able to isolate host cell mutants which enable T4⁺ phage adsorption but not capsid assembly.

Three *E. coli* strains defective for T4⁺ capsid formation have been described²⁻⁴; in these host-defective (*hd*) cells, T4⁺ head production seems to be blocked at an early stage of assembly. Normal T4⁺ head precursor proteins are present but are not processed to the cleaved forms found in the mature capsid structures, and only lumps of head proteins are seen on the bacterial inner membrane. Until now, no direct evidence has been presented to indicate the nature of the host block. Specific T4 mutants in gene 31 are able to overcome this host defect in phage formation. In T431⁻ infection, the major capsid protein accumulates in 'lumps' on the *E. coli* inner membrane⁵, indicating that the product of gene 31 (p31) may affect the association of T4 head proteins with the host cytoplasmic membrane.

The results presented here describe an *E. coli* mutant, *hd*B3-1, that is cold sensitive for T4⁺ head formation. Evidence is presented to show that the defect in *hd*B3-1 is associated with an altered lipid component of the cytoplasmic membrane.

Physiology of T4⁺ infection of *E. coli* *hd* B3-1

E. coli *hd* B3-1 is an N-methyl-N'-nitro-N-nitrosoguanidine derivative of *E. coli* B. Within 10 min after the addition of T4⁺ phage at a multiplicity of infection (m.o.i.) of five phage per cell to mid-log phase *hd* B3-1 bacteria at 30 °C, more than

90% of the cells lose their colony forming ability. *In vitro* complementation tests (data not shown) and electron microscopic studies (Fig. 1) indicate that at 27 °C no detectable T4⁺ capsids are produced in *hd* B3-1, although tails and tail fibres are made in approximately normal amounts. At 27 °C head assembly does not proceed beyond the stage of membrane-bound lumps; bands of detectable T4⁺ head proteins, however, are visible on SDS polyacrylamide gels (Fig. 2). At 41 °C, the infected cells produce an average of 50-80 phage per cell.

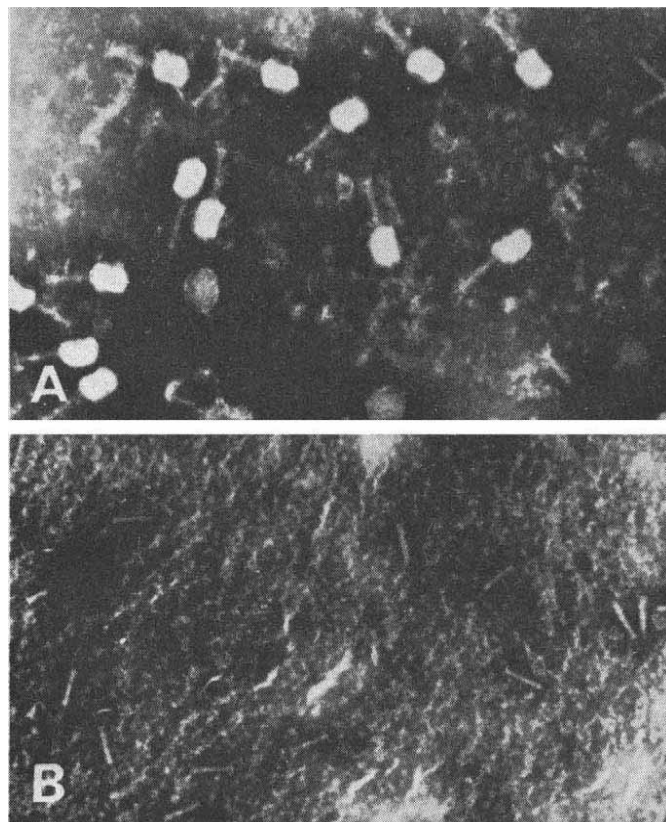


Fig. 1 Electron micrographs of negatively stained crude lysates from T4⁺ infected *E. coli* *hd* B3-1 bacteria. In these and in all subsequent experiments, T4⁺ refers to T4D phages and unless stated otherwise, mid-log phase bacteria growing in L-broth¹ were used. $\times 58,500 \times$. Bacteria were grown and infected at 41 °C with T4⁺ particles at m.o.i. four phages per cell; 5 min later the cells were superinfected with T4⁺ particles (m.o.i. = 4) to inhibit lysis. b, Prepared similarly to a, except temperature was 27 °C.

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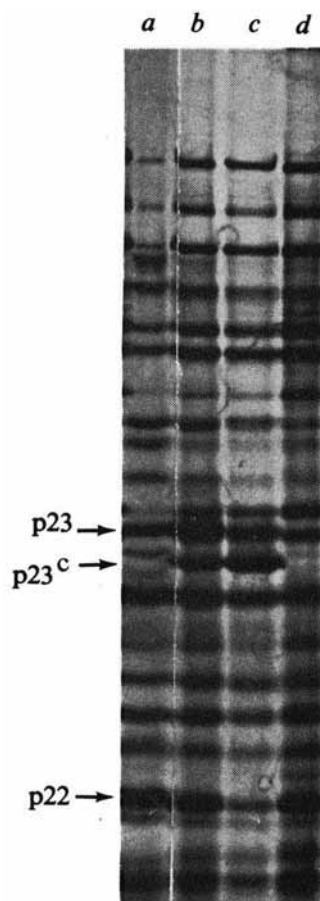


Fig. 2 Autoradiograph of an SDS acrylamide gel showing the phage proteins in $T4^+$ -infected *hd B3-1* cells. *E. coli hd B3-1* and *E. coli B* were grown in LSTG medium (6.4 ml 0.1 M KH_2PO_4 , 1.6 ml 0.1 M Na_2SO_4 , 2.0 ml 0.5 M $MgCl_2$, 100 ml $10\times$ low salts, 100 ml 1.0 M Tris, pH 7.4, 100 ml 20% glucose, H_2O to 1,000 ml; $10\times$ low salts contains 5.4 g NaCl, 3.0 g KCl, 11.0 g NH_4Cl , 10 ml 0.1 M $CaCl_2$, 10 ml 0.1 M $FeCl_3$, H_2O to 1,000 ml). The cultures were then infected (m.o.i. = 3) and superinfected (m.o.i. = 3) 7 min later with $T4^+$ particles or with $T4amH11$ (23^-) mutants. A mixture of ^{14}C -amino acids ($20 \mu Ci ml^{-1}$) and ^{35}S -methionine ($20 \mu Ci ml^{-1}$) was added to the cultures 12 min after infection. The bacteria were pelleted 35 min after infection; the pellets were heated to $100^\circ C$ for 2 min in 1% SDS and 1% β -mercaptoethanol. The denatured proteins were run on SDS acrylamide (10%) slab gels for 5.5 h at 80 V, after which the gels were dried. Autoradiographs were then made to enable visualisation of the protein bands. *a* and *b* *hd B3-1* infected with $T4^+$ phage at 27 and $41^\circ C$, respectively; *c*, *B* infected with $T4^+$ phage. *d*, *B* infected with $T4amH11$ mutant. p23 and p22 are unprocessed capsid proteins. p23^c is the processed form of p23 found in normal $T4$ heads; p22 is degraded during capsid morphogenesis²². Following $T4^+$ infection of *hd B3-1* at $41^\circ C$, p23^c is formed and p22 is degraded, whereas at $27^\circ C$, p23^c does not appear and p22 accumulates.

Intermediate temperatures lead to the production of intermediate numbers of complete phage (Fig. 3). Thus, there is a cold-sensitive block in *hd B3-1* which affects $T4^+$ head assembly.

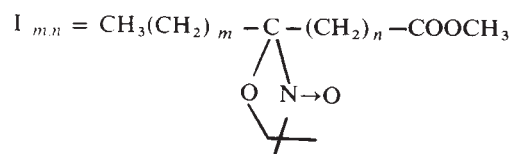
$T4^+$ heads can be found soon after shifting a $T4^+$ -infected *E. coli hd B3-1* culture from restrictive ($30^\circ C$) to permissive ($41^\circ C$) temperature 25 min after infection (Fig. 4). Complete phage appear within 4 min after the shift. Conversely, a temperature shift of $T4^+$ -infected *hd B3-1* cells from 41 to $30^\circ C$ 25 min after infection causes a rapid decrease in phage production (Fig. 4). In other experiments (data not shown) shifting *hd B3-1* cultures from 20 to $30^\circ C$ and from 20 to $41^\circ C$ at the time of $T4^+$ infection, phage capsids were produced only in the cultures shifted to $41^\circ C$. $T4^+$ capsid assembly in *hd B3-1*, therefore, requires temperatures approaching $41^\circ C$, regardless of previous culture conditions.

In addition to *E. coli* strains B and *hd B3-1*, the results reported below include *E. coli B3-1*^{rev}, a spontaneously arising mutant of *hd B3-1* which plates $T4^+$ phages as efficiently as B between 24 and $41^\circ C$. For all experiments the bacteria were growing rapidly, approximately in mid-log phase (4.5×10^8 cells ml^{-1}).

E. coli hd B3-1 inner membrane

As the host cell membrane seems to play a part in $T4^+$ head assembly¹, and as changes in membrane fluidity as a function of temperature may relate to the temperature-dependent *hd B3-1* block in capsid assembly, we suspected that *hd B3-1* may possess an altered cytoplasmic membrane. SDS polyacrylamide gels of inner membranes from *hd B3-1* and B showed identical protein bands. Therefore, we investigated the possible relationship between the host defect and altered inner membrane lipid components.

To determine whether the effect of temperature on $T4^+$ capsid production in *hd B3-1* is related to the mobility of the inner membrane lipids, we used electron spin resonance spectroscopy (ESR). Electron spin-labelled methyl esters of fatty acids



were used to examine the mobility of the lipids of purified⁶ bacterial inner membranes. The membranes were labelled with either 6-(4',4'-dimethyloxazolidinyl-N-oxyl) heptadecanoate ($I_{10,4}$) or 12-(4',4'-dimethyloxazolidinyl-N-oxyl) stearate ($I_{5,10}$); electron spin resonance (ESR) spectra were then

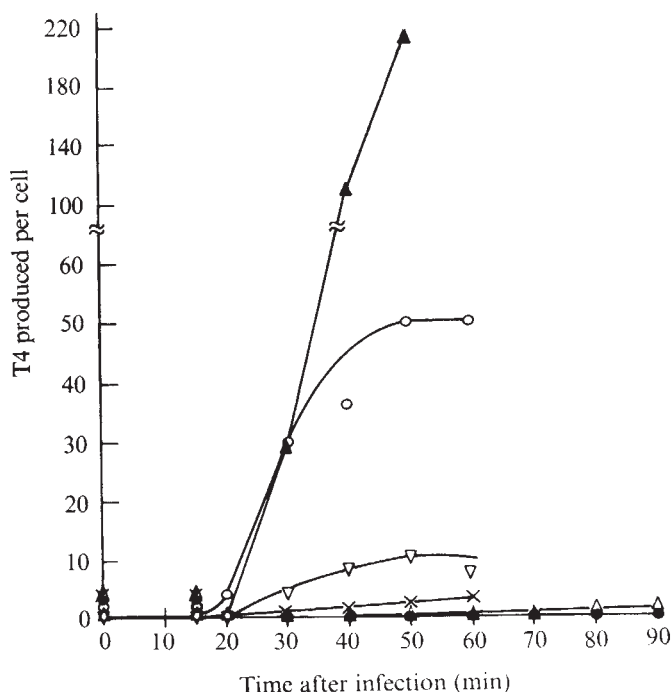


Fig. 3 Phage production following $T4^+$ infection of *E. coli hd B3-1* and *E. coli B*. Bacteria growing at various temperatures were infected with $T4^+$ particles (m.o.i. = 5) and 5 min later they were superinfected (m.o.i. = 5). $\blacktriangle$, B $30^\circ C$; $\bullet$, B3-1 $27^\circ C$; $\triangle$, $30^\circ C$; $\times$, $33^\circ C$; ∇ , $37^\circ C$; $\circ$, $41^\circ C$. Anti- $T4$ serum was added 8 min after infection; 10 min after infection the bacteria were diluted $\times 10^4$ to prevent inactivation of progeny phages by the antiserum. At various times, samples were removed from the infected cultures, exposed to chloroform to lyse the bacteria, and assayed for plaque forming particles to determine phage production.

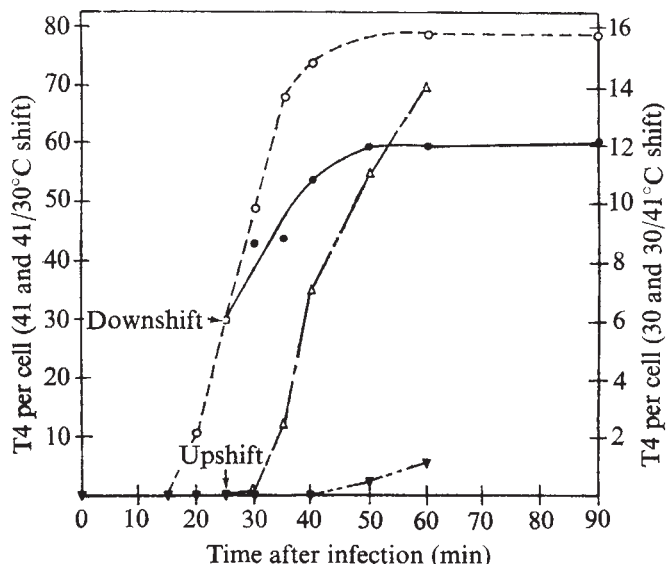


Fig. 4 Effects of temperature shifts 25 min after $T4^+$ infection on phage production in *hd B3-1*. Conditions were similar to those described for Fig. 3, except that 25 min after infection the 30 (∇ , Δ) and 41 ($\circ$, $\bullet$) cultures were each divided into two equal parts; one part was maintained at the same temperature whereas the other part was shifted from the initial temperature of 30 to 41 $^{\circ}\text{C}$ (Δ) or from the initial temperature of 41 to 30 $^{\circ}\text{C}$ ($\bullet$).

obtained over the temperature range 3–60 $^{\circ}\text{C}$. The ratio (B/A) of the mid-field peak intensity to the low field peak intensity was taken as a measure of membrane fluidity. B/A values of nearly 1.0 are characteristic of very fluid, isotropic motion of the spin-labelled fatty acid ester, whereas values greater than 1 typically indicate more restricted motion of the spin-labelled molecule.

A discontinuity in a membrane fluidity profile indicates a transition in the physical state of the spin-labelled fatty acid esters. A discontinuity may indicate true phase transition, that is, a melting of the lipid chains where they are in a solid or ordered state below the transition temperature and are in a fluid or disordered state above the transition temperature⁷. Alternatively, a discontinuity may indicate lateral phase separations of the type described by Shimshick and McConnell⁸ and Linden *et al.*⁹, which involve the clustering of membrane lipids into solid phases, liquid phases and solid-liquid phase mixtures. Lateral phase separations require the occurrence of rapid lateral motion of lipids in the bilayer; this has been observed in several systems^{8,10}.

The data obtained with the $I_{10,4}$ label (Fig. 5a) and with the $I_{5,10}$ label (Fig. 5b) indicate that the purified *E. coli* B inner membranes are more fluid than the *hd B3-1* or *B3-1^{rev}* membranes throughout most of the temperature range. Furthermore, discontinuities are observed in all of the fluidity profiles. With the $I_{10,4}$ label, the *E. coli* B and *hd B3-1* membranes exhibited discontinuities at 38.4 and 48 $^{\circ}\text{C}$, respectively; with $I_{5,10}$ label discontinuities were seen at 31.7 $^{\circ}\text{C}$ for B and at 47 $^{\circ}\text{C}$ for *hd B3-1*. The *B3-1^{rev}* membranes exhibited discontinuities at both 21 and 29 $^{\circ}\text{C}$ with $I_{10,4}$ and at both 19 and 27 $^{\circ}\text{C}$ with $I_{5,10}$. Thus, the temperature at which a discontinuity appears in the membrane fluidity profile is much higher for *hd B3-1* than for either B or *B3-1^{rev}*.

To investigate the chemical bases for these observations, we examined inner membrane phospholipid compositions. Cells were grown for 12 h in medium containing inorganic³²P-phosphate to label membrane phospholipids. Subsequently, inner membranes were purified from the bacteria; the phospholipids were extracted by the procedure of Bligh and Dyer¹¹, and the various phospholipid classes were separated by ascending one-dimensional thin-layer chromatography. Phospholipid spots were identified with iodine vapour, ninhydrin, and molybdate reagent¹².

After labelling for 12 h (Table 1) *hd B3-1* inner membranes had 39% more cardiolipin and 27% less phosphatidylglycerol than B membranes. The *B3-1^{rev}* membranes showed only an 11% increase in cardiolipin and only a 19% decrease in phosphatidylglycerol. The other major phospholipid of the *E. coli* inner membrane, phosphatidylethanolamine, showed little if any change in the different membranes.

To characterise further the membrane lipid components, we investigated the fatty acid compositions of the purified inner membranes. As the fatty acids of the *E. coli* inner membrane are contained in phospholipids¹³, we prepared free fatty acids from the phospholipids by alkaline hydrolysis. Free fatty acids were methylated with diazomethane and separated by gas-

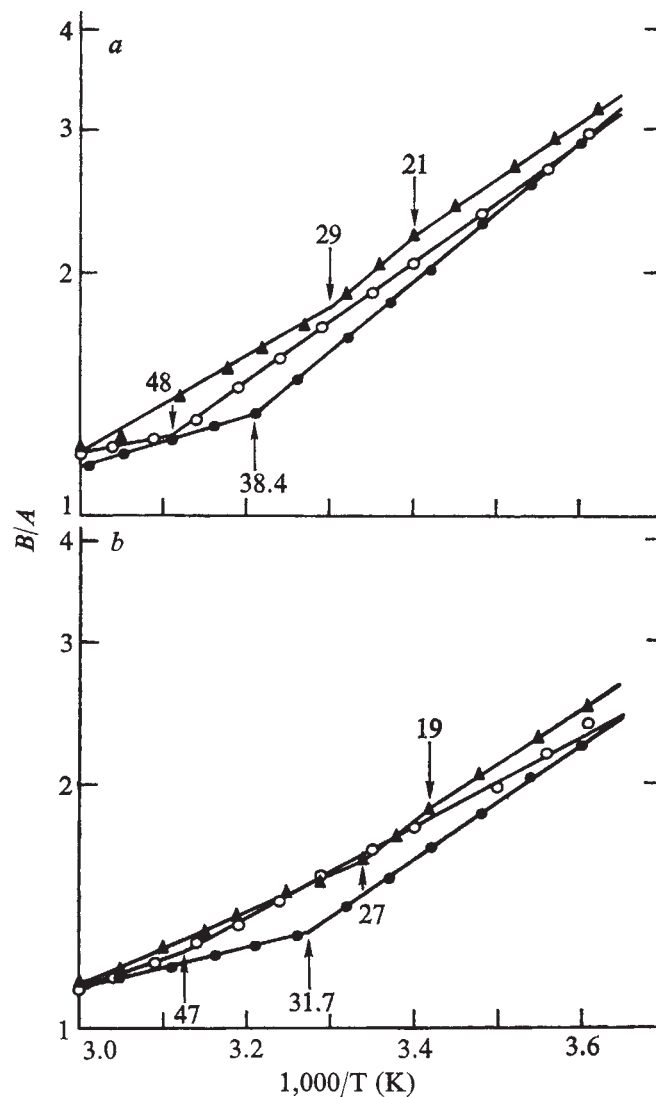


Fig. 5 Temperature dependence of the fluidity (B/A) of spin-labelled fatty acid esters in cytoplasmic membranes from *E. coli* B, *hd B3-1* and *B3-1^{rev}*. Arrows indicate discontinuities in the ESR fluidity profiles. *a*, Spin-labelled fatty acid ester, methyl 6-(4',4'-dimethyloxazolidinyl-N-oxyl) heptadecanoate ($I_{10,4}$) was prepared by the method of Waggoner *et al.*²⁰. *E. coli* inner membranes, purified by the procedure of Osborn *et al.*⁹ from bacteria growing at 37 $^{\circ}\text{C}$, were suspended in 3% sucrose, 10 mM Tris, pH 7.5 at a concentration of 10–12 mg membrane protein ml^{-1} . The procedure for incorporation of the spin-labelled fatty acid esters into the purified membranes has been described¹⁸. ESR spectra were obtained at a frequency of 9,145 MHz on a Varian E4 spectrophotometer equipped with an E4540 variable temperature controller. All samples were 40 μl and were contained in a quartz capillary tube. *b*, Similar to *a*, except using methyl 12-(4',4'-dimethyloxazolidinyl-N-oxyl) stearate ($I_{5,10}$) as the spin label. $\bullet$, *E. coli* B; $\circ$, *E. coli* *hd B3-1*; $\blacktriangle$, *E. coli* *B3-1*.

Table 1 Inner membrane phospholipid composition of *E. coli* B, *hd* B3-1 and B3-1^{rev}

	% Total phospholipids after 12 h ³² P label		
	Cardiolipin	Phosphatidylglycerol	Phosphatidylethanolamine
<i>E. coli</i> B	12.5 ± 0.3	18.3 ± 1.1	69.3 ± 0.9
<i>E. coli hd</i> B3-1	17.3 ± 0.1	13.4 ± 0.3	69.3 ± 0.3
<i>E. coli</i> B3-1 ^{rev}	13.8 ± 0.7	14.9 ± 0.5	71.3 ± 0.9

Phospholipid compositions of purified cytoplasmic membranes from *E. coli* B, *hd* B3-1 and B3-1^{rev}. Cells were grown overnight at 37 °C in tryptone broth (1.0% Bactotryptone, 0.5% NaCl) with ³²P (1.0 µCi ml⁻¹) after which they were diluted 1:500 and grown in fresh tryptone broth with ³²P (1.0 µCi ml⁻¹) for six generations. Phospholipids were then extracted from purified cytoplasmic membranes⁶ by the method of Bligh and Dyer¹¹. One-dimensional, ascending chromatography on Baker Silica Gel IB2 sheets (Baker, Phillipsburg, New Jersey) was used for separation of phospholipid classes with a neutral organic solvent system of CHCl₃-CH₃OH-H₂O (65:25:4, v/v). Phospholipid spots were visualised with iodine vapour, ninhydrin (Sigma, Saint Louis), molybdate reagent¹² and autoradiography. Phospholipid spots were cut from the chromatogram and placed in 5 ml Liquifluor (New England Nuclear, Boston) for determination of radioactivity in a Beckmann LS230 scintillation counter.

liquid chromatography on a diethylene glycol succinate column¹⁴. The proportions of the various fatty acids in our *E. coli* B inner membrane preparations (Table 2) are similar to those reported¹⁵. The proportions of the various fatty acids extracted from *hd* B3-1 inner membranes, however, are distinctly different from those of B (Table 2). Compared with the membranes of B, those of *hd* B3-1 contained 28% more palmitoleic acid (16:1) plus its cyclopropyl derivative, 9, 10-methylenehexadecanoic acid and 18% less *cis*-vaccenic acid (18:1) plus its derivative, 11, 12-methyleneoctadecanoic acid. Palmitoleic acid is known to be the direct precursor of *cis*-vaccenic acid¹⁴. Therefore, the conversion of palmitoleic acid to vaccenic acid seems to be partially blocked in *E. coli hd* B3-1.

Fatty acids extracted from B3-1^{rev} membranes, although containing more 9, 10-methylenehexadecanoic acid, seem to be quantitatively similar to those of B (Table 2). Although there was a 9% increase in palmitoleic acid and methylenehexadecanoic acid in B3-1^{rev}, *cis*-vaccenic acid and its methyleneoctadecanoic acid derivative showed no significant decrease. Comparison of *hd* B3-1 and B3-1^{rev} suggests that the altered fatty acid composition of the bacterial inner membrane and the block in phage capsid production are acquired or lost jointly.

Additional evidence supporting the relationship between the *hd* B3-1 host defect and inner membrane lipid alterations comes from the study of certain *E. coli* B transductants. Cotransduction of markers by P1 phages was used to map the *hd* B3-1 gene responsible for the cold sensitive block. The gene, *fat* A, responsible for the *hd* B3-1 character cotransduced with a proline marker at a very high frequency.

We purified the inner membranes from one such transductant (*E. coli* BOU3101) and from the parental strain (*E. coli* B45) and compared their fatty acid compositions. As T4⁺ capsids fail to assemble in the transductant at 27 °C, we conclude from the data in Table 2 that the *E. coli* BOU3101 transductant acquired the inner membrane fatty acid composition as well as the host-defective character of *hd* B3-1. The membranes from transductants that acquired the proline⁺ gene from B3-1^{rev} showed normal fatty acid compositions. These studies indicate that both the inability of *hd* B3-1 to produce T4 capsids at low temperatures and the inner membrane fatty acid alterations are controlled by the single genetic locus, *fat* A.

T4⁺ phage infection is known to alter the protein and phospholipid compositions of the *E. coli* membrane^{16,17}. To determine whether the differences observed among the inner membranes isolated from uninfected B, *hd* B3-1, and B3-1^{rev} were also characteristic of membranes isolated from similar bacteria 15 min after T4 infection, cultures of B, *hd* B3-1 and B3-1^{rev} (all of which are *su*⁻) were infected with T4amH11 (23⁻) particles. To prevent any differential accumulation of the major capsid protein, p23, on the bacterial inner membranes, T4amH11 phage were used instead of T4⁺; 15 min after infection the cells were chilled and converted to spheroplasts⁶; inner membranes were then purified using the procedure for membranes of uninfected cells.

We again extracted free fatty acids from the membrane phospholipids, after which we determined the proportions of the various fatty acids by gas-liquid chromatography. The

Table 2 Inner membrane fatty acid composition (% of total)

Fatty acid	Uninfected bacteria				
	<i>E. coli</i> B	<i>E. coli</i> B3-1 ^{rev}	<i>E. coli hd</i> B3-1	<i>E. coli</i> B45	<i>E. coli</i> BOU3101
12:0	1.06	0.62	0.38	3.60	1.35
14:0	2.50	2.34	2.94	13.48	7.28
16:0	27.57	29.93	32.00	21.80	32.84
16:1	16.19	9.61	18.75	13.93	29.96
17C	8.15	16.87	12.41	3.91	4.66
18:0	2.15	1.85	1.42	3.73	3.03
18:1	34.90	30.79	28.47	32.26	19.00
UI	(3.38)	(1.48)	(1.10)	—	—
19C	4.05	6.53	2.52	6.29	1.91
UII	—	—	—	—	—
T4amH11-infected bacteria					
Fatty acid	<i>E. coli</i> B	<i>E. coli</i> B3-1 ^{rev}	<i>E. coli hd</i> B3-1		
12:0	2.05	3.25	1.10		
14:0	2.71	4.41	3.13		
16:0	30.68	25.89	31.32		
16:1	17.54	15.20	21.30		
17C	6.56	5.27	9.08		
18:0	1.88	3.47	3.21		
18:1	33.06	29.69	26.31		
UI	(2.14)	(1.27)	(1.57)		
19C	4.46	5.52	1.57		
UII	—	5.81	1.41		

Fatty acid compositions of *E. coli* B, *hd* B3-1, B3-1^{rev}, B45, and BOU3101 inner membrane phospholipids. Inner membranes were purified⁶ either from uninfected bacteria (4.5 × 10⁸ ml⁻¹) growing at 37 °C or from cells which had been infected with T4amH11 (23⁻) particles (m.o.i. = 5). Infected bacteria were centrifuged for 1 min at 12,000g 15 min after addition of phages. Membranes were then prepared in the usual way. The phospholipids of the purified inner membranes were extracted by the method of Bligh and Dyer¹¹. Free fatty acids were prepared from the phospholipids by alkaline hydrolysis¹⁸, followed by immediate methylation with diazomethane. The resultant fatty acid methyl esters were separated on a diethylene glycol succinate column in a Hewlett Packard 5700A gas chromatograph equipped with a chart integrator. Column temperature, 175 °C. N₂ was used as the carrier. Quantitative mixture KD (Applied Science L., State College, Pennsylvania) was used for fatty acid standards. Proportions of the various fatty acids were determined from the areas under the peaks recorded by the chart integrator.

results of this experiment (Table 2) indicate that compared with inner membranes from infected *E. coli* B, the inner membranes from infected *E. coli* *hd* B3-1 cells have a decreased proportion of *cis*-vaccenic plus methyleneoctadecanoic acids and an increased proportion of palmitoleic plus methylenehexadecanoic acids. Furthermore, the membranes from T4^{am}H11-infected B3-1^{rev} show nearly the same fatty acid composition as the control. These data are very similar to the data obtained from uninfected bacteria.

Implications

The above data indicate that purified inner membranes from *E. coli* *hd* B3-1 bacteria have altered fatty acid and phospholipid compositions as well as an unusually high viscosity. Further, studies of transductants for the host-defective character and of spontaneous revertants suggest that both the cold-sensitive block in T4 head production and the inner membrane alterations are controlled by the same gene, designated *fat* A. As T4⁺ heads assemble on the *E. coli* inner membrane¹, we conclude that the cold sensitivity of T4⁺ capsid formation in *hd* B3-1 is caused by the altered lipid components of the inner membrane.

Cotransduction by P1 phage, rather than conjugation, was used to map the *fat* A locus as numerous attempts to make *hd* B3-1 male (F⁺) failed. The parent strain, *E. coli* B, was readily made F⁺ in control experiments. It seems that, in addition to its effect on T4 morphogenesis, the *hd* B3-1 *fat* A membrane lipid alteration blocks a step in the acquisition or expression of the F episome, both at low (30 °C) and at high (41 °C) temperatures.

The *fat* A locus, responsible for the *hd* B3-1 phenotype, seems to be near proline in the *E. coli* B chromosome. Genes coding for proteins involved in fatty acid uptake and degradation are also closely linked to proline²¹. The genetic determinants of the host-defective phenotypes of two other *E. coli* strains in which T4⁺ capsid production is blocked^{2,3} are located near melibiose, far from proline. Therefore, two bacterial genes separated by nearly one-fifth of the *E. coli* chromosome can affect T4 head formation. Both of these genes, however, seem to affect the same cellular structure because T4 mutants in the same gene—gene 31—can overcome either, and possibly both, bacterial blocks. The product of T4 gene 31 functions during the organisation of T4 head proteins on the bacterial membrane⁵. An interesting hypothesis to account for these observations is that the initiation of T4 head assembly

requires specific protein-binding sites on the *E. coli* inner membrane. Capsid production could be blocked either by altering this membrane protein (genetically linked to melibiose) or by changing membrane lipid components (linked to proline) associated with the protein, so that at low temperatures formation of the capsid protein-membrane protein complex would be sterically or allosterically inhibited. At higher temperatures the increased disorder of the membrane lipids might enable the initiation of capsid assembly. An example of this type of effect of membrane lipids on the interaction of a membrane protein with a cytoplasmic molecule is the cold sensitivity of sheep kidney ATPase activity¹⁸. Changes in the organisation of the cytoplasmic membrane lipids seem to be responsible for the marked reduction in the rate of ATPase activity which occurs below 20 °C.

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β structures of alternating polypeptides and their possible prebiotic significance

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A survey of the commonest amino acids formed in prebiotic conditions suggests that the earliest form of genetic coding may have specified polypeptides with a strong tendency to form stable β-sheet structures. Poly(Val-Lys), like other polypeptides in which hydrophobic and hydrophilic residues alternate, tends to form β structures. We show that bilayers with a hydrophobic interior and a hydrophilic exterior may be present in aqueous solution.

MILLER and others have published a large number of papers dealing with the prebiotic synthesis of amino acids¹. The formation of polypeptides from amino acids in prebiotic conditions has also been reported¹. Since we know a great deal

about the conformational tendencies of the naturally occurring amino acids², it should now be possible to deduce the probable conformations of the polypeptides first formed on the primitive Earth.

The assignment of amino acids to trinucleotides by the genetic code is not random. Woese has emphasised that the position of an amino acid in the codon-table is strongly dependent on its hydrophobic or hydrophilic character³. Dickerson has suggested that the codon assignments correlate with the tendency of amino acids to occur in the interior or on the surface of globular proteins⁴.

Here we bring together conformational information obtained with synthetic polypeptides, information about the abundance of prebiotic amino acids, and the suggestions of Woese and

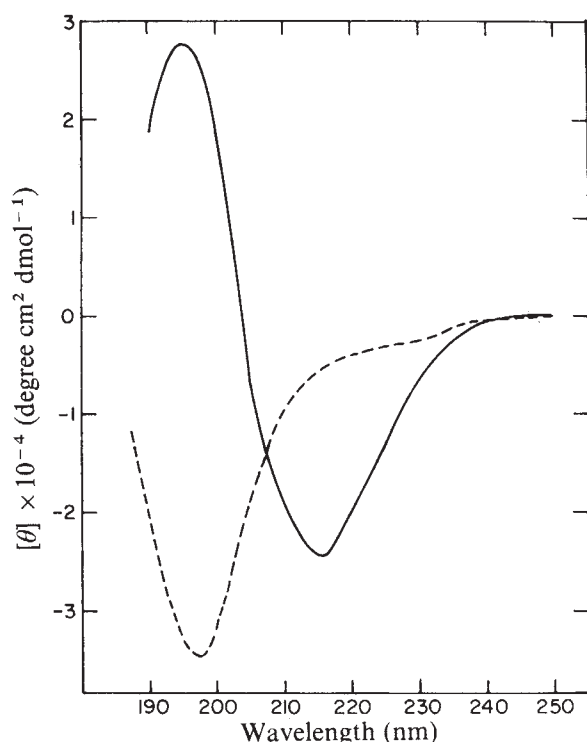


Fig. 1 CD spectra of a freshly prepared solution of poly(Val-Lys) at pH 2.3 in water (---) and of a solution in 0.1 M NaCl after 60 h (—) at pH 2.3.

Dickerson in an attempt to throw light on the origin of the genetic code. In particular, we provide further support for the suggestion that peptides with alternating hydrophilic and hydrophobic residues, that form β -sheet bilayers, may have been important at an early stage in the origins of life⁵.

Alternating hydrophobic-hydrophilic residues

A large number of sequential copolypeptides has been synthesised (for a review see Johnson⁶). Among those soluble in water and containing alternating hydrophobic and hydrophilic residues, we are aware of only four that have been submitted to conformational analysis. Three of these polypeptides exhibited the β structure in aqueous solution.

Alternating poly(Glu-Ala) shows a typical β -circular dichroism spectrum after standing for several weeks in neutral aqueous solution⁷. Poly(Tyr-Glu) forms soluble aggregates below pH 10.5. Infrared spectroscopy shows that these aggregates have the antiparallel β structure⁸. Seipke *et al.*⁹ made a detailed study of the conformation properties of poly(Lys-Phe). At neutral pH and low salt concentration the polymer is partly in the β form. The proportion of β form is markedly increased at basic pH, or when sodium perchlorate or methanol is added to the solution. The corresponding random copolypeptide poly(Lys, Phe) exhibits a random coil conformation at neutral pH, and changes to the β form at higher pH, but sodium perchlorate induces a transition to the α helix, as Peggion *et al.* had already shown¹⁰. The remarkable difference in the behaviour of the two polymers shows that the amino acid sequence can dramatically change the tendency to form a β structure.

Two silks that contain a substantial proportion of alternating residues have been studied in the solid state. It has been shown that the crystalline β region of *Bombyx mori* silk is made up of chains of the type (Gly-X)_n where X is alanine or occasionally serine. In the structure established by Marsh *et al.*¹¹ the glycyl residues are located on the same side of the sheets and the packing of successive sheets bring glycyl residues in contact, thus leading to an alternation of glycyl-glycyl and alanyl-alanyl contact layers. Such a structure is also adopted by synthetic poly(Ala-Gly)¹².

Sawflies of the family Argidae produce a silk that contains

roughly equimolecular amounts of alanine and glutamine, accounting for 70–80% of the amino acids. The X-ray diffraction patterns provide evidence that the β structure of this silk is very similar to that found in *Bombyx mori* silk¹³.

Conformational analysis of poly(Val-Lys)

Poly(Val-Lys) with a molecular weight of about 5,000 was obtained by polymerisation of the *p*-nitrophenyl ester of the corresponding protected dipeptide. The optical and chemical purity of the sample was confirmed. Details of the synthesis will appear elsewhere.

At acidic pH in pure water the CD spectrum of poly(Val-Lys) (Fig. 1) is almost the same as that of random coiled *Bombyx mori* silk fibroin in aqueous solution¹⁴. We suppose, therefore, that poly(Val-Lys) is a random coil in these conditions. When a 1% or 0.01% acidic solution was brought rapidly to pH 8.8 a spectrum typical of the β conformation was obtained. The transition was almost reversible after 15 min at pH 8.8. The polymer precipitated from solution above pH 9.

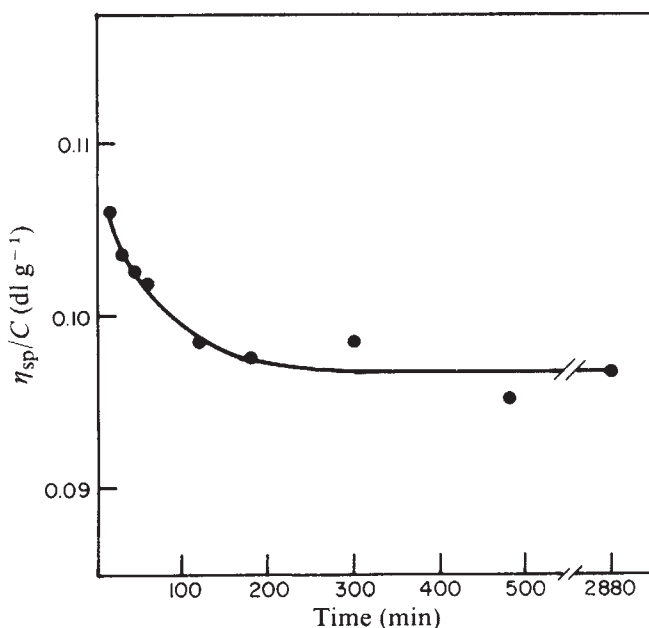
When sodium chloride was added to a 1% solution of the polymer at pH 2.3 to bring the final NaCl concentration to 0.1 M (the polymer cannot be dissolved in this salt solution) a slow coil $\rightarrow$ β transition occurred and was almost complete after 60 h (Fig. 1). There was no transition in the absence of added salt, even after one week.

Addition of urea (6 M) to a 1% solution in 0.1 M NaCl at pH 4.0 did not prevent the appearance of the β structure, but reduced the rate of the coil $\rightarrow$ β transition. Guanidine hydrochloride (0.5–6 M) caused the precipitation of the polymer as a β structure at pH 4.4.

The reduced viscosity of a 1% solution in 0.1 M NaCl at pH 2.2 was measured as a function of time. We found a significant decrease of the viscosity with time during the first 5 h (Fig. 2). No change was induced by increasing the pH to 7.8 after the solution had equilibrated at pH 2.2 for 48 h (Fig. 3). Above pH 7.8 the viscosity increased sharply. The appearance of a light turbidity indicated that the polymer begins to precipitate in these conditions.

The meniscus depletion technique was used to determine the apparent molecular weight distribution of the polymer in a 0.05% solution in 0.1 M NaCl at pH 5.5, while it was undergoing its coil $\rightarrow$ β transition. At low speed (12,590 r.p.m.), a first equilibrium was reached which produced molecular weights of about 200,000. At higher speed (59,780 r.p.m.), a molecular weight of 5,200 was measured.

Fig. 2 Time dependence of the reduced viscosity of 1% poly(Val-Lys) in 0.1 M NaCl at pH 2.3.



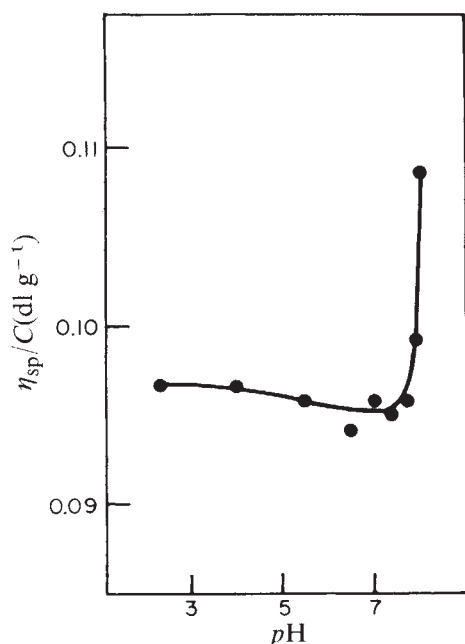


Fig. 3 pH dependence of the reduced viscosity of an aged 1% solution of poly(Val-Lys) in 0.1 M NaCl.

The polymer, in the disordered or the β conformation, gave a solution that frothed readily. The surface tension was measured with a Langmuir–Wilhelmy balance. Pure water (72 erg cm⁻²) and L- α -dipalmitoyl lecithin (0 erg cm⁻²) were taken as standards. Surface tensions were 30 erg cm⁻² and 25 erg cm⁻² for fresh acidic solutions and aged solutions in 0.1 M NaCl at pH 3.0, respectively.

Solid state

Dialysis of an aged solution in 0.1 M NaCl at pH 2.3 (β form) against 0.5 M sodium perchlorate precipitated the polymer. The product exhibited the characteristic infrared absorption bands of the β structure (amide I band at 1,630 cm⁻¹ with a shoulder at 1,690 cm⁻¹ indicating an antiparallel arrangement of the chains). The powder pattern of the X rays showed 10 lines, 4 of which were well defined at 26.2 ± 0.5 Å, 8.62 ± 0.6 Å, 4.65 ± 0.03 Å and 3.82 ± 0.02 Å. X-ray studies on the β structure of polyvaline have been reported¹⁵, but without any details. We obtained a powder pattern with strong lines at 9.2 and 4.57 Å, which can be interpreted as the intersheet distance and the interchain distance, respectively.

Implications of conformational studies

The experimental observations described in the previous sections make it clear that the chains of poly(Val-Lys), like those of poly(Lys-Phe), adopt a β conformation in a variety of conditions. We found no evidence for the formation of an α helix, whereas the random coil was stable only in acidic solutions in the absence of added salts.

We do not have any direct experimental evidence as to the spatial organisation of the polypeptide chains into β sheets in solution. We believe, however, that indirect arguments suggest strongly that the bilayer structure shown in Fig. 4c is present rather than sheets of the types shown in Fig. 4a and b.

A striking feature of the X-ray powder photograph of the perchlorate salt of poly(Val-Lys) is the strong reflection at 26.2 Å. Analogy with the diffraction patterns obtained from other β structures¹⁶ shows that this reflection must correspond to the repeat distance perpendicular to the plane of the sheets. The reflection at 4.65 Å corresponds to the usual interchain distance, which commonly lies between 4.62 and 4.78 Å (ref. 16). The reflections at 8.62 and 3.82 Å probably correspond to the 003 and (210+211) reflections, respectively, in an orthorhombic unit cell with $a = 4.65$, $b = 26.2$ and $c \sim 6.8$ Å.

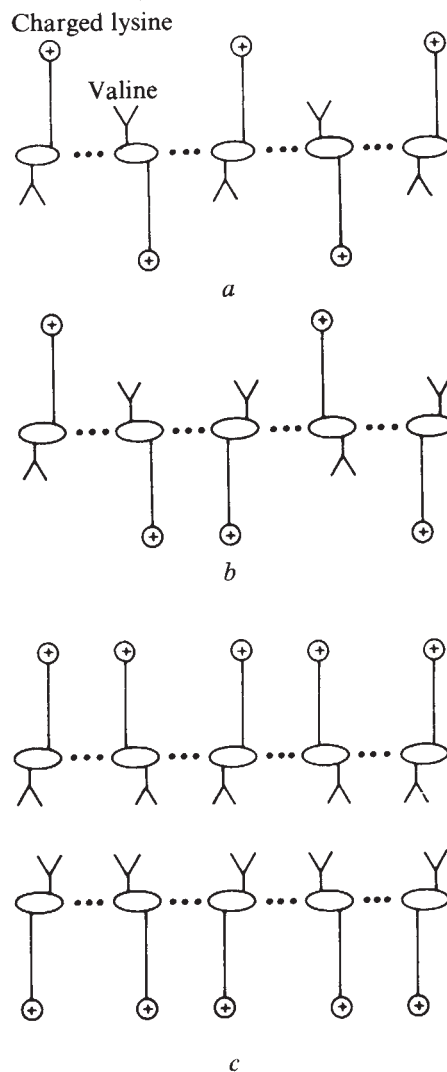
If we assume this unit cell, it is possible to find a distribution of the atoms in the ac plane which predicts a weak 002 reflection, and an intensity ratio for the 001 and 003 reflections close to that observed. The X-ray powder pattern is, however, too poor to enable us to confirm the proposed unit cell.

The phosphate salt of polylysine forms a β -sheet structure with an intersheet distance of 17.16 Å (ref. 17). Our own measurements on the β structure of polyvaline give an intersheet distance of 9.2 Å. If we suppose that the structure illustrated in Fig. 5a would have a repeat equal to the sum of the repeats in the phosphate of polylysine and in polyvaline, we arrive at a value of 26.4 Å, in good agreement with our measured value. On the other hand, the repeat distance for the β -sheet packings illustrated in Fig. 4a and b or for the structure shown in Fig. 5b would be much smaller, probably less than 17 Å. We believe that the X-ray data strongly support a stacked bilayer structure in the solid state and, by inference, a similar bilayer structure in solution.

The behaviour of the polymer in solution is consistent with this interpretation. At pH 2.2, in the absence of salt, poly(Val-Lys) exists as a random coil. The addition of small amounts of salt induces a transition to a β structure. This is most simply interpreted as due to the shielding effect of the salt which enables the positively charged amino groups to come together on one side of a β sheet.

The precipitation of the polymer by larger amounts of salt can be explained in a similar way. The formation of a stacked-bilayer structure involves the bringing together of sheets of

Fig. 4 Diagrammatic representation (in chain axis projection) of various arrangement of poly(Val-Lys) chains in β sheets. Ellipses represent the backbone.



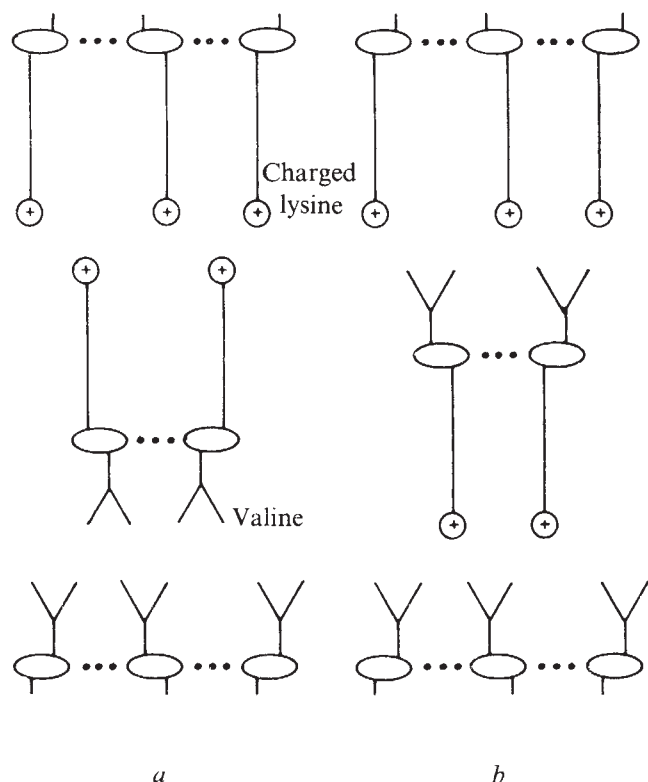


Fig. 5 Diagrammatic representation of two packings of β sheets.

positively charged residues. This is only possible if salt is present in high enough concentration to provide the necessary shielding. The precipitation that occurs in more alkaline solutions (pH 8) is made possible by the neutralisation of the charges on a proportion of the lysine residues.

The high molecular weight (200,000) of the polymer in the β sheet form shows that extensive aggregation has occurred, but throws no light on the spatial arrangement of molecules in the aggregate. Similarly, the drop in viscosity that occurs when the random coil is converted to an equilibrium β sheet in acidic solution gives no detailed structural information.

The β structure which is formed reversibly when freshly-prepared solutions are shifted from acidic to alkaline conditions must be different from the structure formed irreversibly by ageing in acidic solution. Unfortunately, we do not have any evidence as to the detailed structures. We cannot, for example, distinguish between normal β structure and cross β structures.

The surface activity of poly(Val-Lys) is consistent with our hypothesis. At an air-water interface, we should expect the polymer to form a β -sheet monolayer, with the lysine residues penetrating into the aqueous layer and the valine residues exposed to the air. It is also possible, however, that the β structure is disrupted at the interface and that the surface activity is due to the formation of micelles.

The fact that diverse alternating polypeptides form a β structure at all, also provides strong evidence favouring the bilayer structure. It is hard to see what factor other than hydrophobic interaction between residues confined to one side of a β sheet, could account for the general tendency to form β sheets. The formation of a β structure by poly(Lys-Phe), in conditions in which the corresponding random copolymer forms an α helix or a random coil, is particularly striking.

Prebiotic polypeptides

A survey of many experimental studies of the amino acids formed in prebiotic conditions shows that the two simple α -amino acids, glycine and alanine, are formed more readily than any others. In addition, serine, threonine, aspartic acid, glutamic acid and a variety of aliphatic amino acids (including

many that are non-biological) are formed in small and variable amounts. Some α -amino acid derivatives, particularly N-methyl amino acids, and some α -hydroxy and β -amino acids, are also formed. All potentially optically active compounds are obtained as racemic mixture¹.

We do not know if any substantial fractionation of the α -amino acids in the prebiotic mixture took place before condensation of polypeptides occurred. If we suppose that this was not the case, then the first prebiotic condensation products are likely to have been copolymers of glycine and DL-alanine, containing substantial amounts of other α -amino acids. The first peptides might have contained larger amounts of the higher aliphatic amino acids, if enrichment of hydrophobic amino acids was important, or of serine, threonine and the dicarboxylic acids, if hydrophilic amino acids were concentrated.

The conformation of polypeptides containing glycine and alanine have been studied. It is clear that random peptides derived from a mixture of these amino acids would not contain much α helix, if any, since glycine is a strong helix breaker. On the other hand, it seems likely that β -sheet formation would be considerable¹⁸. The incorporation of N-methyl- α -amino acids and hydroxy acids into the polypeptides would decrease the extent of β -sheet formation, but we are justified in assuming that, in so far as prebiotic 'polypeptides' formed structures at all, rather than random coils, they formed β -sheet structures.

This conclusion is strengthened by the observation that in those cases where a peptide can form either α helices or β sheets, the latter structure is favoured if the molecular weight of the polypeptide is low¹⁹. Since uninterrupted sequences of α -amino acids in prebiotic condensation products are likely to have been short, β -sheet structures would have had a further advantage over α -helices.

Alternating peptides and the genetic code

The amino acids which are specified by codons with U in the central position—Phe, Leu, Ileu, Met, Val—are all hydrophobic, while those specified by codons with A in the central position—Glu, Asn, Gln, His, Lys, Tyr—are all hydrophilic. The situation is less extreme for the two other groups, but amino acids specified by codons with central C—Ser, Pro, Thr, Ala—tend as a group to be somewhat more hydrophobic than those with a central G—Gly, Ser, Arg, Cys, Trp. Tryptophane and possibly cysteine are exceptions to this rule^{3,4}.

Studies of mononucleotide-polynucleotide interaction strongly suggest that, in the absence of enzymes, it would be difficult to replicate polynucleotides in which purines are adjacent. Thus polynucleotides in which purines alternated with pyrimidines are likely to have been abundant in the prebiotic soup.

On the basis of these two observations we suggested⁵ that the first form of genetic coding 'translated' polynucleotides in which purines and pyrimidines tended to alternate into polypeptides in which hydrophobic and hydrophilic amino acids tended to alternate. Finally, we suggested that alternating polypeptides of this kind were important because they tended to form β sheets with one hydrophobic and one hydrophilic surface. Such sheets would stack together to form 'membrane-like' aggregates. The reader is referred to our previous paper⁵ for an amplification of the arguments summarised in this section.

Experimental evidence obtained since our original publication confirms the prediction that alternating polypeptides tend to form β sheets. Although this by no means proves that our hypothesis concerning the first step in the origin of the genetic code is correct, it suggests that it may be worth looking further for a structural basis for the code. Perhaps the code first assigned glycine to codons with a central purine and L-alanine to codons with a central pyrimidine, while L-serine was associated with both types of codon. This would have generated a crude 'silk'. It may be significant that the amino acids that tend to occur in β turns in globular proteins² are coded for by triplets of the form PuPuPy or PyPyPu. We have so far been

unable to provide a convincing explanation of other major features of the genetic code.

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letters to nature

Energy spectrum of hadrons in cosmic rays at sea level

BARUCH *et al.*¹ have measured the energy spectrum of hadrons (nucleons and pions) in cosmic rays at sea level and have presented evidence for an anomalous behaviour in the energy range 2–8 TeV. For $E < 2$ TeV they find the differential vertical intensity to decrease with increasing energy as $E^{-2.6 \pm 0.1}$, for $2 < E < 8$ TeV the intensity is almost constant, and for $E > 8$ TeV the intensity again decreases with increasing energy (Fig. 1). The 'step' in the spectrum for $2 < E < 8$ TeV is unexpected and Baruch *et al.* propose that it is produced by the interaction of a new particle, of rest mass 40–70 GeV c^{-2} , mean lifetime $> 2 \times 10^{-7}$ s and an interaction length of $1,000^{+1,000}_{-700}$ gcm⁻². We have made new measurements on the hadron spectrum at the higher energies in question.

The experimental arrangement used consisted of, from top to bottom; a layer of 15 cm of lead; a plastic scintillator (area 1 m²); eight layers of neon flash tubes; 15 cm of iron; a further plastic scintillator (area 1 m²) and 116 layers of neon flash tubes. Hadrons interacting in the lead and iron absorbers produce pions and on the average one-third of these are neutral. Each neutral pion decays into two γ rays and the ensuing electron photon cascade emerges from the absorber and traverses the scintillators. Detailed calculations have been made of the relationship between the average number of burst particles below the absorber and the energy of the initiating particle. The result can be represented approximately by the statement that the mean energy of the 'primary' which produces N particles is N GeV—this relationship is valid over most of the energy range in question; the difference in burst size for incident protons and pions is not large, the size being about 30% larger for pions than for nucleons in iron and 20% larger in lead. The observation of large voltage pulses from either scintillator was used as a master trigger and the pulse heights were displayed on an oscilloscope trace after being delayed by 0.3 μ s and 0.9 μ s. Subsequently a high voltage pulse was applied to the flash tubes and a photograph showing the geometry of the burst was obtained. Data were taken using different trigger levels and also two different time delays (20 μ s and 330 μ s) between the occurrence of the burst and the application of the high voltage pulse to the flash tubes. The longer time delay was used for the large burst sizes ($N > 400$ particles), so that it was possible to define the axis of the burst

more precisely—the point being that with such large bursts the total number of particles is very large, as is their lateral spread, and only by reducing the flash tube efficiency can the axis be determined accurately.

All the flash tube layers contained parallel tubes and in the front view bursts were only accepted if their axes passed through a well defined geometrical region and made an angle of $\pm 30^\circ$ to the vertical. The maximum angle a burst could make with the vertical in the back plane was 81° and 75° for the lead and iron targets respectively. Using the method of Lovati *et al.*² the measured projected angular distribution of bursts was used to determine the spatial angular distribution, assuming it to be of the form $I(\theta) = I(0) \cos^2 \theta$. In this way both n and the vertical intensity $I(0)$ of the burst spectrum were determined. For bursts of size above 400 particles n was found to be 8.0 ± 1.0 , in agreement with the expected value.

Conversion from burst size to mean hadron energy was made assuming that all the particles are nucleons; in fact some are pions for which the conversion factor will be slightly different as mentioned already but the ratio of pions to nucleons is not expected to be a rapidly varying function of energy so that the important spectral shape should not be distorted. High energy muons also produce bursts but the effect is small. For a burst size corresponding to 5 TeV hadrons (the centre of the Baruch *et al.* step) the effect is 5% for the lead and $< 1\%$ for the iron.

The final hadron spectrum has been found by averaging the burst spectra from the lead and iron targets (the individual spectra were compatible, adding confidence to our conclusions) and the resulting energy spectrum is shown in Fig. 1 where the hadron spectrum from the measurements of Baruch *et al.*, is also plotted. The rates shown in the figure are absolute rates and no normalisation has been made to other work. Our results show no evidence for a step in the energy range 2–8 TeV and they are in fact consistent with a spectrum of the form $E^{-2.7 \pm 0.1}$ over the whole energy range from 10 GeV to 10 TeV. There could indeed be an irregularity in the form of the spectrum which would escape detection in this experiment because of the smoothing introduced by the method, by the fluctuations in burst size. To assess the effect of fluctuations we have calculated the probability that a primary proton produces N burst particles per unit N . Assuming the hadron spectrum to be of the form given by Baruch *et al.*, we then calculated the shape of the expected burst spectrum. The result was that the step was clearly present in the burst spectrum. We are therefore convinced that we would have detected the

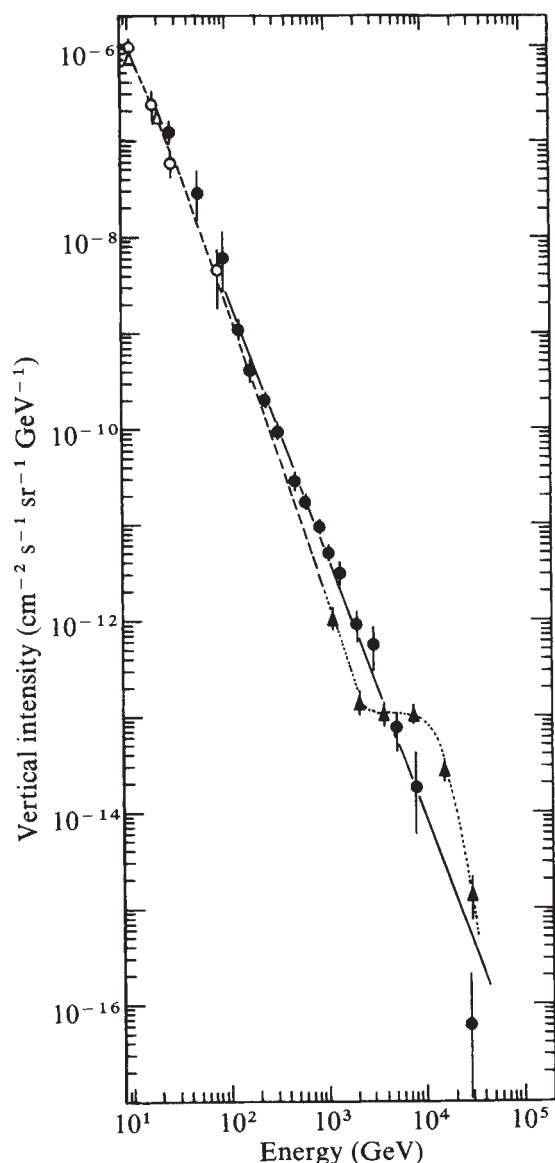


Fig. 1 The differential energy spectrum of hadrons (and of nucleons alone) in cosmic rays at sea level. The low energy data (≥ 100 GeV) are estimates of the total nucleon spectrum found by multiplying the measured proton intensities of Brooke and Wolfendale³ and Diggory *et al.*⁴ by a factor 2 (assuming equal numbers of protons and neutrons). The dashed line is an estimate of the total nucleon spectrum found by increasing the measured neutron intensities summarised by Ashton⁵ by a factor of 2. The solid line is the best fit to the present measurement of the energy spectrum of all hadrons. The difference between the energy spectrum of all hadrons (solid line) and the energy spectrum of all nucleons (dashed line) is due to the increasing contribution of charged pions to the total hadron flux for energies > 100 GeV. Δ , Hadrons¹; $\bullet$, hadrons (our work); $\circ$, nucleons³; $\triangle$, nucleons⁴.

bump if it was a real effect.

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Thermal model of oceanic lithosphere

THE thermal model for oceanic lithosphere developed by McKenzie¹ assumes that oceanic plates are slabs of constant thickness. The model has been used successfully to reproduce both the heat flow and the topographic patterns observed in oceanic lithosphere². There is, however, no reason to assume that the lithosphere has a constant thickness; indeed, it is likely to thicken as it moves away from the ridge and cools.

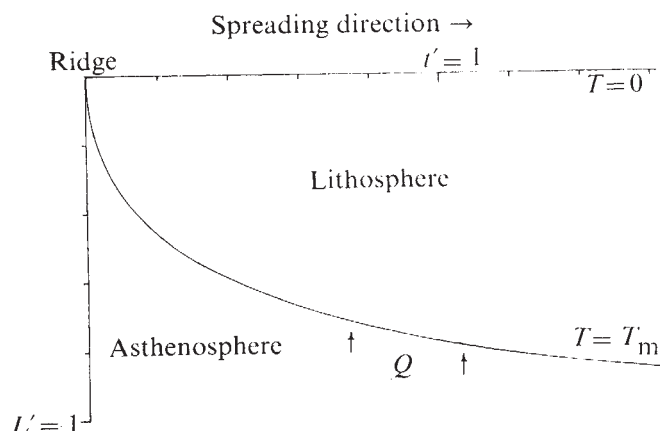
Thermal models have been derived^{3,4} which predict that the thickness, L , of a section of lithosphere should be proportional to the square root of the time, t , since its formation at the ridge, and experiments using spreading layers of wax have confirmed this relationship⁵. Such a model also agrees with the available seismic data (D. Chapman and H. Pollack, personal communication).

Theoretical considerations, using the best estimates for material parameters, predict, however, that the lithosphere should thicken much faster than the available evidence suggests it actually does. This theoretical rate of thickening can be reduced if the effects of the latent heat of the asthenosphere's partial melt are taken into consideration, but to achieve thicknesses equal to those actually observed the asthenosphere must be assumed to be completely liquid³. We show here that realistic results can also be obtained if it is assumed that there is a heat flux into the base of the oceanic lithosphere from the underlying asthenosphere.

We have obtained an approximate solution for the temperature field and the thickness of the lithosphere by modifying McKenzie's model. If the lithosphere is viewed in two-dimensional cross section (Fig. 1) it rests on the low-velocity zone, which is taken to be a nearly isothermal zone of partially molten material at a temperature T_m . Except for thin surface layers, the lithosphere is identical to the material beneath it but colder and, therefore, more rigid. The base of the lithosphere is taken as the T_m isotherm. The material beneath the lithosphere is assumed to be capable of maintaining a uniform heat flux, Q , to the base of the lithosphere by convection. The upper surface of the lithosphere is maintained at 0°C by sea water. At the ridge crest, hot, mantle material accretes to the plate margin. As this material moves away from the ridge it is cooled by sea water. The slab conducts heat away from the mantle beneath it, causing material to freeze on to the bottom of the lithosphere. At equilibrium, the lithosphere attains the proper thickness such that it loses heat upwards as fast as it receives heat from the mantle beneath.

As the lithosphere is very long compared with its thickness and because the temperature difference between its top and

Fig. 1 Predicted shape of the lithosphere plotted in dimensionless variables (see text).



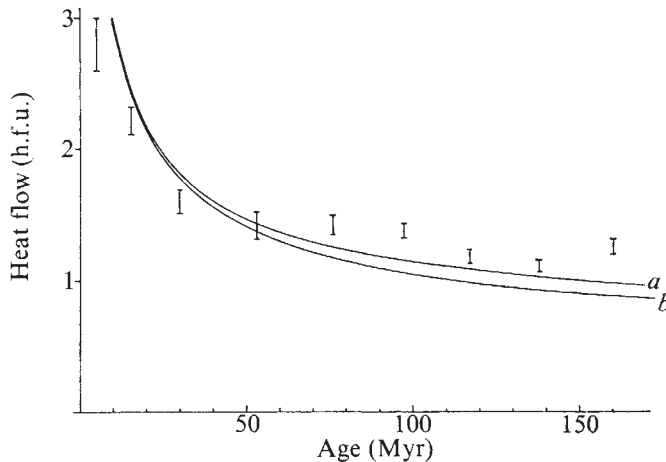


Fig. 2 Observed variation (I) of surface heat flow with age for the northern Pacific² compared with theoretical predictions (—). (a, $Q = 0.8$; b, $Q = 0.56$). The error bars indicate the estimated error of the calculated mean and are much smaller than the true scatter of observed values.

bottom surfaces is large, the effects of horizontal heat conduction can be ignored, and the thermal history of a single column of lithosphere as it cools in time can be considered. Assuming that material properties are constant and that there are no radioactive heat sources, the governing heat flow equation is

$$\rho C_p \partial T / \partial t = k \partial^2 T / \partial z^2$$

where T is temperature, z is depth, k is thermal conductivity, ρ is density, and C_p is heat capacity. The boundary conditions are that $T = 0$ at $z = 0$, $T = T_m$ at $z = L(t)$, and $k \partial T / \partial z = Q$ at $z = L(t)$. An approximate solution to this equation can be obtained using a technique developed to handle boundary layers in fluid mechanics. It is assumed that the temperature at any time within a column of lithosphere can be represented by a third order polynomial in z .

$$T(z, t) = a + (bz/L) + (cz^2/L^2) + (dz^3/L^3)$$

where a , b , c , d , and L are functions of time. Considering the boundary conditions together with the fact that $\partial T / \partial t = 0$ at $z = 0$ then $\partial^2 T / \partial z^2 = 0$ at $z = 0$ means that the coefficients can be determined

$$T(z, t) = (3T_m z / 2L) - (Qz / 2k) - (T_m z^3 / 2L^3) + (Qz^3 / 2kL^2) \quad (1)$$

For $Q = 0$ this function is a good approximation to the error function which is the exact solution for this case. At equilibrium the temperature distribution in the lithosphere will be linear in z such that $kT_m/L = Q$, so L will equal kT_m/Q . Substituting this value of L into equation (1) the approximation of the temperature distribution becomes linear as L approaches its equilibrium value.

From equation (1) the heat flow out of the top of the lithosphere at any time is found to be

$$k \partial T / \partial z |_{z=0} = (3kT_m / 2L) - (Q/2) \quad (2)$$

Neglecting radioactivity, this heat must come from either a deeper source or from the cooling of the slab. At any time, the heat content of a column of lithosphere is

$$\rho C_p \int_0^L T(z) dz = \rho C_p [(3T_m z^2 / 4L) - (Qz^2 / 4k) - (T_m z^4 / 8L^3) + (Qz^4 / 8kL^2)] |_{z=0}^L \quad (3)$$

If the slab increases its thickness by ΔL , the original thickness L would have a different, smaller heat content, given by equation (3), with $L + \Delta L$ substituted for L in the denominators of the fractions. The difference between the two values, which is the heat liberated by increasing the thickness of the lithosphere, is $\rho C_p [(3T_m/8) + (QL/4k)] \Delta L$.

In the time interval Δt the heat budget of an individual column of lithosphere is balanced if

$$[(3kT_m/2L) - (Q/2)] \Delta t = \rho C_p [(3T_m/8) + (QL/4k)] \Delta L + Q \Delta t$$

or, in the limit

$$dL/dt = (4/\rho C_p) [(kT_m/L) - Q] / [T_m + (2QL/3k)] \quad (4)$$

The latent heat of the melt solidified to the base of the slab could be included in this balance without difficulty. It is omitted here because if the fraction of partial melt in the asthenosphere is small then its latent heat has a small effect compared with those of the other terms in the equation.

If $Q = 0$, equation (4) has the solution

$$L = (8kt/\rho C_p)^{1/2} \quad (5)$$

which is of the form of previous thickening models. This is also the relationship observed in wax experiments in which there is no deep source of heat.

With Q arbitrary, equation (4) has the solution

$$(5/3)(\ln[1/(1-L')]) - L' - L'^2/3 = t' \quad (6)$$

where L' and t' are dimensionless variables

$$L' = LQ/kT_m \quad t' = tQ^2/k\rho C_p T_m^2$$

and an arbitrary constant has been fixed so that $L = 0$ at $t = 0$. This solution is plotted in Fig. 1. For small values of

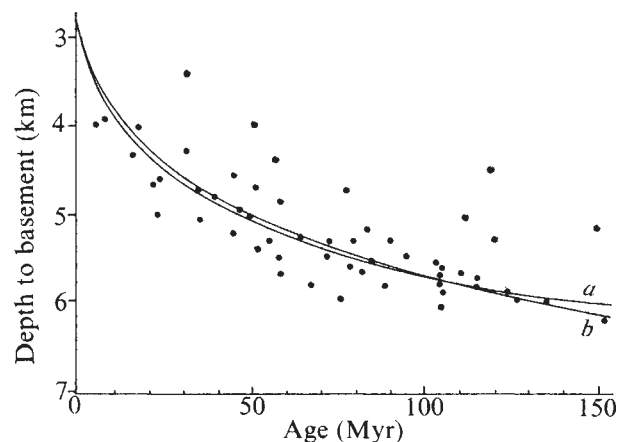


Fig. 3 Observed variation the depth of ocean basement with age⁹ compared with theoretical predictions. Data are from all available oceans. ●, Depth determined by drilling; —, prediction (a, $Q = 0.8$; b, $Q = 0.56$).

L' , when the contribution from Q is minor, equation (6) reduces to equation (5) as would be expected. As $t' \rightarrow \infty$, $L' \rightarrow 1$, so $L \rightarrow kT_m/Q$ as wanted.

Two numerical examples can be calculated using generally accepted values for material parameters: $T_m = 1,200^\circ\text{C}$; $C_p = 0.25 \text{ cal/g } ^\circ\text{C}$; $\rho = 3.3 \text{ g/cm}^3$; $k = 7 \times 10^{-8} \text{ cal/cm s } ^\circ\text{C}$. For estimates of Q , two values obtained from continental heat flow studies are used: $Q = 0.8 \text{ h.f.u.}$ ($0.8 \times 10^{-6} \text{ cal/cm}^2 \text{ s}$) represents a probable upper limit to the actual sub-lithospheric flux⁶; $Q = 0.6 \text{ h.f.u.}$ has been suggested as a value compatible with estimates of the heat production of the lower crust and upper mantle². To represent this latter value we use $Q = 0.56 \text{ h.f.u.}$ because it leads to

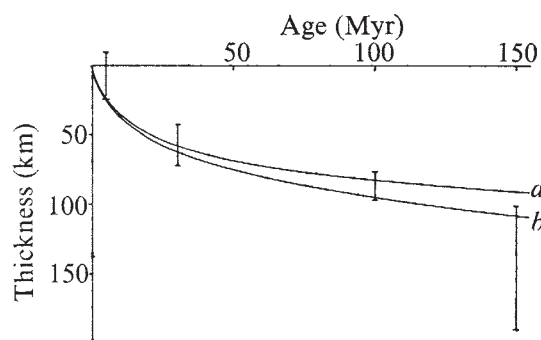


Fig. 4 Comparison of lithospheric thickness (derived from surface wave studies in the northern Pacific)¹⁰ with theoretical predictions. I Seismic estimate; —, prediction (a, $Q = 0.8$; b, $Q = 0.56$).

convenient scale factors. With Q equal to 0.8 h.f.u., $L = L' \times 105$ km and $t = t' \times 100$ Myr. For Q equal to 0.56 h.f.u., $L = L' \times 150$ km and $t = t' \times 200$ Myr. The theoretical heat flow curves which result from these material values give a good fit to the data (Fig. 2). Both curves are lower than the data from the older age range, but the difference is only 10–20%. The theoretical curves can probably be raised by that amount if latent heat and radioactivity effects are included in the model.

A theoretical subsidence curve can be calculated by assuming that isostatic compensation takes place at the base of the lithosphere⁷. The mass of any column of water and lithosphere at any time must then equal the mass of the same height of water plus asthenosphere at the ridge. So

$$s(t) = L(t)(\rho_L - \rho_A)/(\rho_A - \rho_W)$$

where $s(t)$ is the subsidence of the surface of the lithosphere below the height of the ridge crest, ρ_W and ρ_A are the densities of seawater and asthenosphere, respectively, and ρ_L is the mean density of the lithosphere. For the case $Q = 0$, ρ_L is constant so $s(t)$ is proportional to $L(t)$. For $Q \neq 0$, the mean density of the lithosphere can be calculated from equation (1) using the assumption that density change results entirely from thermal contraction⁷. The factor $(\rho_L - \rho_A)/(\rho_A - \rho_W)$ becomes $\rho_0 \alpha T_m [(3/8) + (L'(t)/8)] / (\rho_0 - \rho_W)$ where α is the coefficient of thermal volume expansion and ρ_0 is the density at 0 °C. To calculate subsidence curves ρ_0 is taken to be 3.3 g cm⁻³ and α is adjusted so that the subsidence equals 3 km at 100 Myr (Fig. 3). The fit to the available data is quite good. For $Q = 0.56$ h.f.u., α must equal 4.0×10^{-5} °C⁻¹, for $Q = 0.8$ h.f.u. it must equal 4.5×10^{-5} °C⁻¹. The measured expansion coefficient for olivine at high temperatures⁸ (400–800 °C) varies from 3.1 – 4.4×10^{-5} °C⁻¹, in agreement with the calculated results.

Seismic surface-wave determinations of the depth to the low-velocity zone as a function of the age of oceanic crust¹⁰ are in reasonable agreement with the theoretical predictions (Fig. 4). The error bars in Fig. 4 do not indicate the total uncertainty, but only the allowable variation within the constraints of a preferred model of velocity structure. It is possible that the large thickness calculated to have been obtained after 150 Myr is not real.

As well as providing a physically realistic way of explaining existing heat flow, topographic, and seismic data, this thermal model offers a possible resolution of the oceanic–continental heat flow problem. The surface heat flux in the older oceanic areas is approximately the same as the surface heat flux in the older continental shield areas. Previously, it has been assumed that the older oceanic areas are in thermal equilibrium², an idea which was incompatible with the observed near equality of heat flux. As the oceans have much less surface radioactivity than the continents, then if they have the same flux as the

continents at depth they should have a much lower flux at the surface. Our results, however, indicate that the older oceans are not in thermal equilibrium. With a sub-lithospheric heat flux of 0.8 h.f.u., the surface flux at 100 Myr is predicted to be 45% higher than the equilibrium flux. With a sub-lithospheric flux of 0.56 h.f.u., the surface flux at 100 Myr is 85% higher than at equilibrium. Thus, the approximate similarity of surface heat flux in ocean basins and continental shields may be purely coincidental.

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Seawater near the head of the Ross Ice Shelf

THE Ross Ice Shelf (Fig. 1) is a sheet of ice that floats over 540,000 km² of the Ross Embayment¹. The ice in the southern part is between 300 and 800 m thick, and floats on as much as 400 m of water^{2,3}. Until now any knowledge of the water mass beneath the shelf has been inferred from studying the open water of the Ross Sea far to the north. Here, I describe a newly discovered natural access to seawater beneath the Ross Ice Shelf, 550 km south of the shelf front and 770 km from the South Pole, on the north-eastern margin of an ice rise.

The ice rise is an elongate north-westerly-trending dome about 40 km long which reaches an elevation of 100 m. The ice

Fig. 1 Location of the rift containing seawater (a) near the head of the Ross Ice Shelf, Antarctica. A–A', Section line for Fig. 4; b, graben; c, crevasse pattern; d, sledging route; e, ice cliffs 15–20 m high.

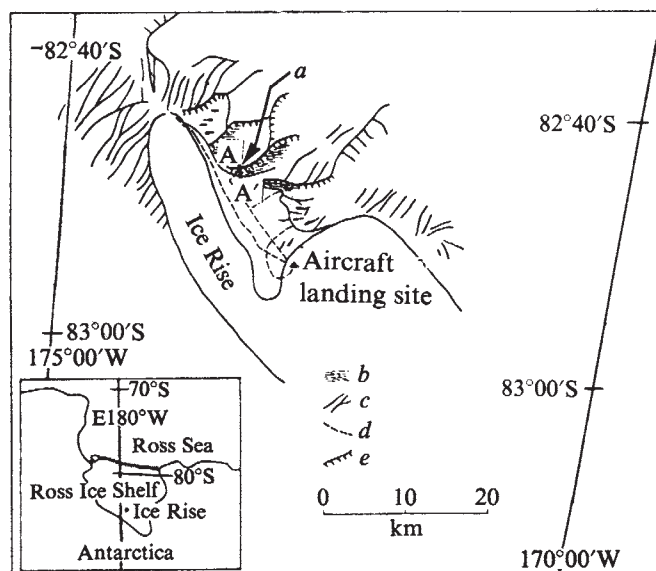


Table 1 Salinity* and freezing point† determinations and cation concentrations‡ for seawater samples from rift shown in Fig. 1

	Conductivity ratio	Salinity (‰)	Freezing point (°C)	Cation concentration (‰)			
				Na	Mg	Ca	K
Sample A							
Subsample a	1.0002	35.01					
b	0.9980	34.92					
c			-1.92				
			-1.98				
Sample B							
Subsample a	0.9945	34.78					
b	0.9931	34.73					
c				9.63	1.12	0.31	0.45

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†Dr A. DeVries, Biology Laboratory, McMurdo Station, Antarctica.

‡Mr P. Kyle, Victoria University of Wellington, New Zealand. Values determined by atomic spectroscopy (error, $\pm 10\%$).

of the rise is grounded² and the northern and eastern margins are marked by extensive crevassing that continues for many kilometres north of the rise. This crevassing is believed to reflect a flow regime of shearing and extension as the floating shelf moves past the rise on its journey north. Along the north-eastern margin of the rise the ice shelf has broken into plateau-like blocks of ice several kilometres across, separated by broad valleys floored with snowed-in, broken ice ('horst' and 'graben' structures). The southern margin of each block is a continuous

I placed a marker in the ice at sea level to check this (Figs 2 and 3). For the first 6 h the water level did not change more than 1 mm relative to the ice, but 24 h later the ice had risen 40 mm relative to the water level. Further markers were placed and measured on several occasions, though, following a blizzard, not all could be relocated for the final measurements.

The measurements are accurate to 2 mm, and establish two points. First, no tidal cycle is detectable from markers on either side of the rift. Because the tidal range is large enough to be measured directly it is concluded that the ice on both sides of the rift is floating (see Fig. 4). Second, the data also show that the rift zone is highly mobile. All four stations on the northern side, and two on the southern side, rose more or less steadily over a five day period at rates of between 38 and 65 mm d⁻¹; the other two stations rose less than 29 mm over the five day period (Fig. 3). Two strain measurement triangles across the rift between stations 3 and 4 showed that the rift widened by 0.23 and 0.27 m over the 77-h period between 1000 on December 8 and 1500 on December 11, 1974. If these high rates of movement were maintained for more than a few tens of days the ice-floored rift would be many metres across and the margins of the rift would rise metres above the valley floor. Neither feature has, in fact, been observed along the rift, and it is therefore concluded that both the horizontal and the vertical movements form part of a long term 'glaciotectonic'

Table 2 Deuterium concentrations of water in the rift

Sample	δ_{SMOW}^*	Sample description†
CRL-5	-18‰	Surface water from near Station 1 (Fig. 2)
CRL-6	-11‰	Surface water from near Station 4 (Fig. 2)
	-6‰	Sea water beneath ice in McMurdo Sound, south-western Ross Sea (Hole 3) ⁹
	-250‰	Snow at Scott Base, McMurdo Sound‡

* δ value in parts per 10³ deviation from Standard Mean Ocean Water (SMOW) ($\pm 3\%$).†All samples were vacuum distilled before analysis and were analysed using the hot probe method⁸.

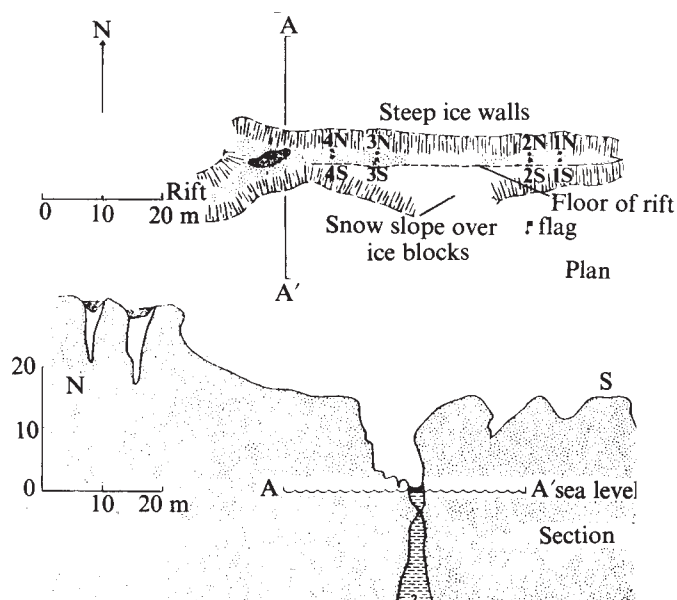
‡G. L. Lyon, personal communication.

ice cliff about 15 m high (Fig. 1); the northern margin is both less regular and more snow-covered than the southern margin. At the foot of one of the cliffs a narrow rift extends down to a thin ice floor with water beneath; chemical (Table 1) and isotopic (Table 2) analyses have shown that this is seawater.

The rift can be traced for more than 2 km along the foot of the ice cliffs, and it probably extends along their entire length. For a 50-m-long section of the rift (Fig. 2) the maximum width of the floor is 2 m, and its thickness is about 0.1 m. Several soundings through holes in the ice indicated an irregular underwater topography. The greatest depth plumbed at the eastern end of the rift was 2.9 m, and at the western end was 4.9 m. The 'feel' of the line indicated, however, that the surface was rough, hard and sloping, and not bottom. Soundings from a wider part of the rift should reach much greater depths.

The only sign of life along the rift is a yellowish brown patchy staining, which is common up to about 0.5 m above the water level on the northern side. The staining proved to be the remains of algae. Filamentous wisps as much as 80 mm long are abundant in the water under ledges and beneath the ice floor of the rift. Samples of the material include living organisms containing traces of fresh pigment (H. W. Johnstone, personal communication). The samples consist largely of the disrupted cell debris of centric diatoms, with some intact frustules, and a few whole pennate diatoms. Disruption probably resulted from the freezing of the samples after collecting, for temperatures in the field ranged from -3 to -10 °C.

I had expected to be able to observe the tide, known to have a range of between 1.0 and 1.5 m over this part of the shelf⁴, and

Fig. 2 Sketch plan and section of the rift containing seawater, showing locations of the eight pegs measured for Fig. 3.

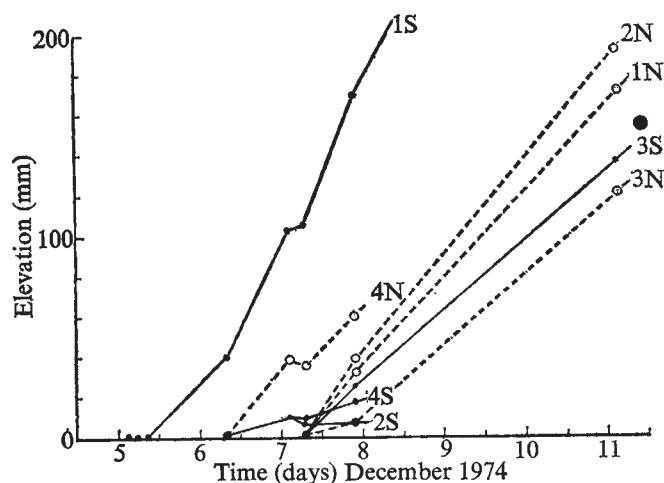


Fig. 3 Vertical movement of pegs on the north and south sides of the rift floor. Peg locations shown in Fig. 2. ●, South side; ○, north side.

cycle, with a period probably in the range 10–100 d. These local movements are believed to relate to the regional ice flow pattern, but further and more accurate measurements are necessary to check this.

I conclude that the rift is filled with water in free circulation with the Ross Sea. The salinity is slightly higher than Antarctic surface water or circumpolar deep water, but lies within the range of Ross Sea shelf water⁵ (34.75 ‰ –> 35.00 ‰). Furthermore, the presence of living algae indicates a direct connection with the waters of the open Ross Sea 550 km to the north. The

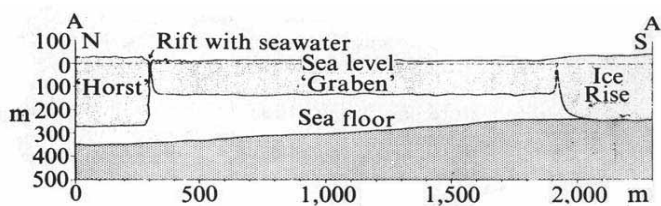


Fig. 4 Cross-section north from the edge of the ice rise to a block of the Ross Ice Shelf (A–A' in Fig. 1), showing underwater ice and bottom topography inferred from surface elevations with respect to sea level.

normal marine salinity indicates that there is little freezing or melting in the rift, or at least that the circulation is sufficient to prevent a measurable change in salinity. The mean annual temperature is -27°C (ref. 6), but the temperature gradient, and thus the rate of freezing or melting, of ice forming the rift is not known.

The discovery of seawater near the head of the Ross Ice Shelf provides a topographic datum (sea level) that was not previously available in the region. The rift itself also provides biologists with access to an unusual new ecosystem. The fishing prospects are quite good too, for specimens of the benthic fish *Trematomus bernacchii* have recently been caught beneath permanent ice cover 100 km from the open sea in the Antarctic Peninsula⁷. Experience suggests that crevassed areas on ice shelves are worth closer examination. In the past they have been regarded as hazards to be avoided, but it is now evident that some crevasses have considerable scientific value as natural access ways to the sea beneath.

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Cryptic suture in the eastern Grenville Province

IN order to explain palaeomagnetic data from the Precambrian Shield of North America (Laurentia), Irving *et al.*¹ have suggested that Grenvillia was separated from Interior Laurentia 1,150 Myr ago and then rejoined it about 1,000 Myr ago to form the present day Canadian Shield. This hypothesis requires that a suture exists within the Grenville Province south of a region of Grenville rocks which are considered to be metamorphosed equivalents of rocks within the adjacent, older structural provinces, and north of sampling sites used to determine Grenvillia poles (Fig. 1); the Grenville Front has been discounted as the site of the suture¹. A weakness of this hypothesis is that there is no known geological evidence for a suture in the appropriate location. It has been suggested¹, however, that the crust now exposed as the Grenville Province originally occupied deep crustal levels (10–20 km) and that any traces of former intervening oceans may have been destroyed. We present here an interpretation of gravity data in the region which is in accord with the presence of a cryptic suture within, or proximal to, the eastern part of the suture zone as delineated by the palaeomagnetic investigations.

A Bouguer anomaly map of the eastern Grenville and neighbouring regions (Fig. 1) shows a striking, linear negative anomaly, the Grenville Front Low², extending from Lake Mistassini to the Labrador coast, a distance of about 1,200 km. For the whole of this distance the anomaly straddles the Grenville Front and its axis lies consistently within the Grenville Province. In profile (Fig. 2) the anomaly is asymmetrical with gradients on the northern flank consistently averaging about $0.35 \text{ mgal km}^{-1}$, and those on the southern flank maintaining an average of about 1 mgal km^{-1} . The linearity of the southern flank is locally disturbed by anomalies associated with large gabbroic intrusions. The background level of anomalies north of the Grenville Front Low is noticeably lower by about 15 mgal (ref. 2) than that over the Grenville Province.

Various hypotheses—massive granites³, a metasedimentary trough⁴, and a crustal root combined with low velocity–low density seismic layers⁵—have been advanced to explain the anomaly, but strong evidence in favour of one or other of these hypotheses is largely lacking². The anomaly is clearly the expression of large scale, crustal structure and not a manifestation of discrete lithological variations. Tanner⁶ has suggested a model comprising a denser than 'normal' Grenville upper crust in contact with Superior crust of 'normal' density; the remaining portions

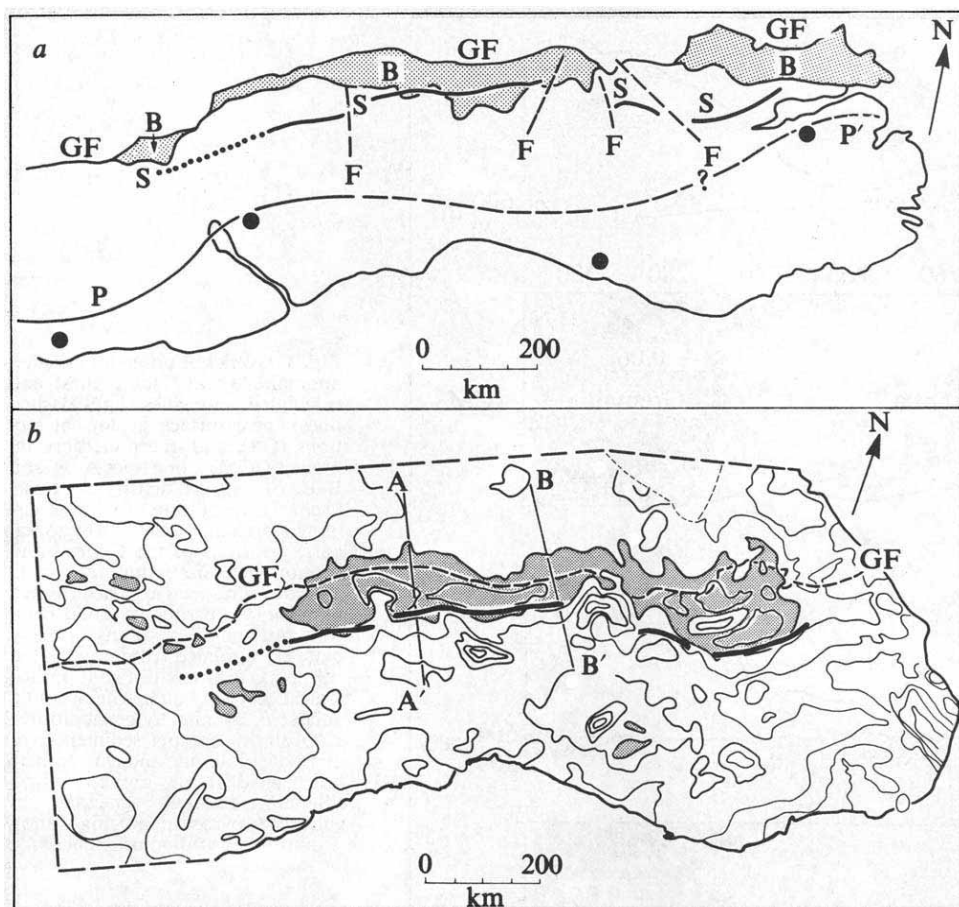


Fig. 1 *a*, Outline map of eastern Grenville Province: B (shaded areas), terrain B of Irving *et al.*¹; GF, Grenville Front; P-P', northern limit of palaeomagnetic poles obtained for Grenvillia; dashed lines (F), interpreted faults; heavy solid lines (S), trace of suture interpreted from gravity anomalies; dotted line (S), trace of suture interpreted from magnetic anomalies; ●, sampling sites for palaeomagnetic studies¹. *b*, Bouguer anomaly map of eastern Grenville Province; contour interval, 20 mgal; shaded areas, anomalies with values of less than -80 mgal; position of suture outlined with same line pattern as in *a*; GF, Grenville Front; A-A', B-B', lines of gravity interpretations illustrated in Fig. 2.

of both crusts are of 'normal' density. On this model the compensating base of the thicker Grenville crust would be the principal cause of the low and of the gentle northern flank of the anomaly, and the denser upper crust, dipping about 45° south-eastwards from near the Grenville Front to a uniform depth of 20 km, would explain the steep southern flank and the higher background level of anomalies over the Grenville Province. The thicknesses of the Superior and Grenville crusts (35 and 40 km, respectively⁶) have been confirmed seismically⁷.

Seismic work⁷ has also indicated a two-layer crust for both structural provinces, with the Conrad discontinuity occurring at depths of 14 and 21 km, respectively, under the Superior and Grenville provinces; a thicker crust (45 km) with the Conrad discontinuity at a depth of 24 km was indicated under the axis of the Grenville Front Low. Using the seismic information Thomas⁷ proposed a model in which the gentle northern flank of the anomaly results from the downward flexuring of the older structural provinces towards the axis of the anomaly. The effect of such downwarping is to induce lateral density changes between the upper and lower crusts and between the lower crust and the mantle, thus producing mass deficiencies. Supporting evidence for such flexuring is provided by the preservation of the string of Aphebian and Helikian sedimentary-volcanic basins along the northern side of the Grenville Front. The seismic root⁵ may also contribute to the gravity low. The steep flank is formed by a major break separating a denser Grenville crust from Superior crust. This postulated break occurs up to 100 km south of the Grenville Front, thereby placing the front itself entirely within Superior-type crust. We suggest that this break is the line of the cryptic suture.

Gravity interpretations for a single layer crust along the lines A-A' and B-B' of Fig. 1 are shown in Fig. 2. The

models are extremely simple and in each case comprise Superior-type crust (34 km thick) in contact with Grenville crust (39 km thick) which is 0.06 g cm⁻³ denser; the density of the mantle is 0.4 g cm⁻³ greater than that of the Superior crust. Under the axis of the anomaly the Superior-type crust attains a thickness of 38 km, but this value falls short of the 45 km predicted from the seismic analysis⁵. It is impossible to reconcile gravity and seismic thicknesses without changing the density of the lower crust under the axis of the gravity anomaly. The contact between the two crustal blocks in both models slopes southwards under the Grenville Province, from points near the lower portion of the southern flank of the anomaly; these points are located 37 km (A-A') and 55 km (B-B') south of the Grenville Front. On the basis of these interpretations it has been possible to sketch the surface trace of the suture (Fig. 1) which is extended south-westwards along a line separating areas of strongly contrasting magnetic patterns⁷.

Evidence of a major, continuous structural feature has not yet been recognised on the surface along the predicted trace. We believe, however, that this feature represents a cryptic suture of the type presented in the continental collision-basement reactivation model of Dewey and Burke⁸ (such as the Indus Suture). The widespread distribution of anorthosites and granulite facies metamorphism within the Grenville Province is compatible with this model for the crustal levels exposed at present. At these levels it is likely that ultramylonites and pseudotachylites, which are believed to occur in the deeper levels of sutures⁸, will ultimately be discovered in the region of the proposed suture. Dewey and Burke⁸ believe that the analogue of the Indus Suture for the Grenville situation lies south-east of the exposed Grenville Province. Our model suggests, however, that suturing has occurred along the northern edge of the Grenville Province which concurs with the hypothesis

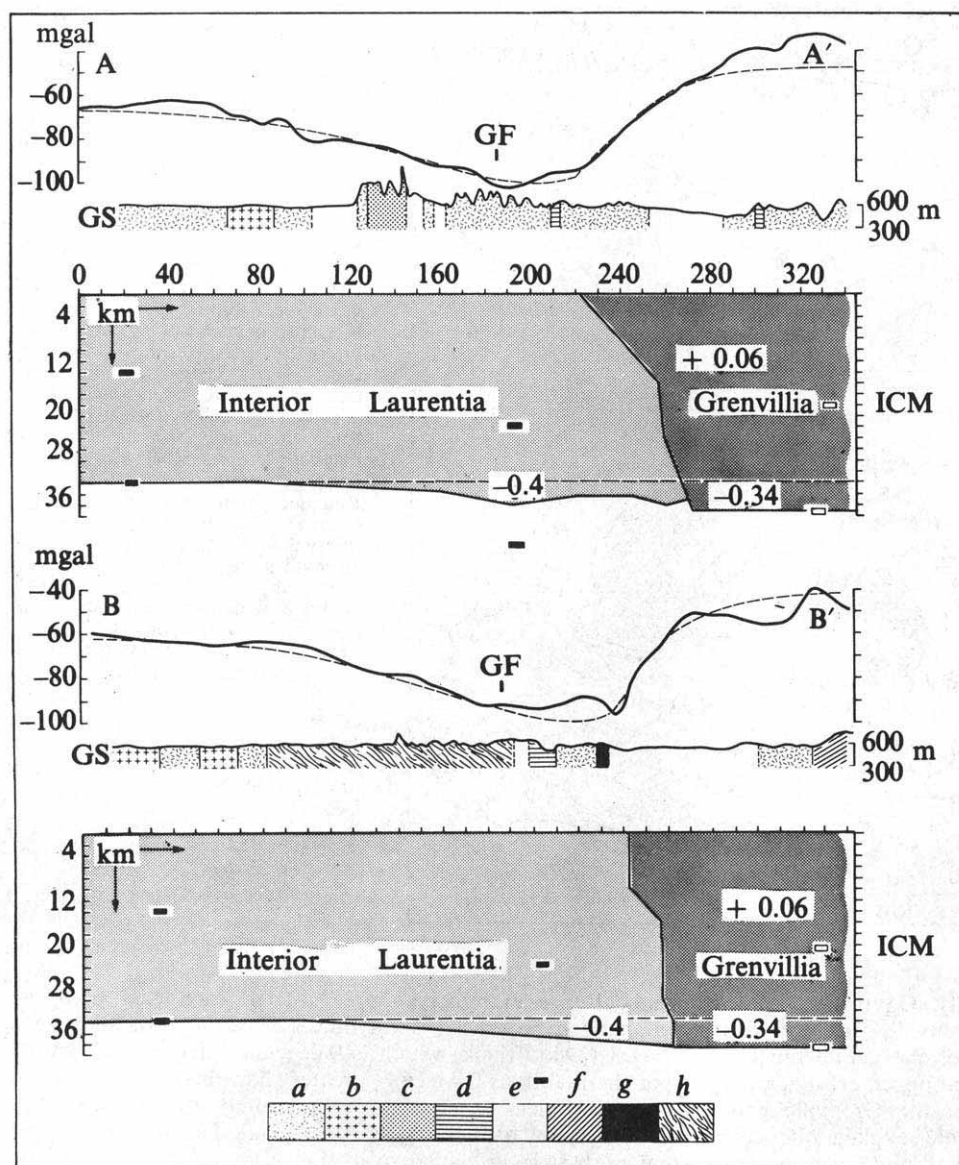


Fig. 2 Profiles of observed Bouguer anomalies (heavy solid lines) and calculated anomalies (light dashed lines), near-surface geological sections (GS), and interpreted crustal models (ICM) along lines A-A' and B-B' of Fig. 1; density contrasts (g cm^{-3}) are indicated for the interpreted crustal models. Horizontal bars, positions of the Conrad and Mohorovicic discontinuities (upper and lower, respectively) determined by seismic experiments (solid bars, true horizontal positions; hollow bars, extrapolated from outside the region); GF, Grenville Front. Lithological key: a, quartzofeldspathic gneiss; b, granite to granodiorite; c, Otish Mountains sediments; d, quartzite, dolomite and iron-formation; e, drift or unmapped; f, sillimanite-bearing gneiss; g, gabbro; h, pyroxene-bearing granodiorite and granodiorite gneiss.

of Irving *et al.*¹. Subduction would have occurred in this case from north-west to south-east.

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Design of organic metals and superconductors

I WISH to draw attention to the potential of odd alternant hydrocarbons (OAHs) in the design of organic metals and superconductors which gives reason to suppose that they may prove equal or even superior to present systems. There is now strong evidence for the existence of the metallic state in

certain organic charge transfer salts, and a suggestion of low temperature superconducting fluctuations in the case of tetrathiofulvalene-tetracyanoquinodimethane (TTF-TCNQ); present interest is now focused on the stabilisation of these states at greater (critical) temperatures¹. In contrast to the molecular crystals usually formed by conjugated organic molecules (which are held together by van der Waals' forces and characterised by a relatively weak intermolecular interaction), these salts crystallise as segregated one-dimensional stacks of cation and/or anion radicals in which the charge transfer process and thus the electronic mobility within a stack is greatly enhanced by the abnormally high overlap of the π -electron wave functions of nearest neighbours in the stack and by the ability of the constituent molecules to stabilise an ionic fluctuation.

Odd alternant hydrocarbons are described in refs 2 and 3; it will be sufficient to note that within the Huckel molecular orbital (HMO) theory, neutral OAHs possess one unpaired electron in a formally non-bonding molecular orbital, and as alternant hydrocarbons have zero charge density at all atoms. It also follows that an OAH can give rise to a stable, closed shell cation and anion (in which there is effective dispersal of charge density) and that the bond orders (and by inference bond lengths) remain invariant among this triad of oxidation states. Furthermore, the discussion will depend on the extrapolation of the properties of the isolated molecules into the crystalline state. This assumption is likely to provide a reasonable description of the solid state properties, as these one-dimensional

charge transfer compounds give rise to a single narrow conduction band (the bandwidth of which is proportional to the intermolecular electron-transfer integral (t)), and may be studied in the tight-binding approximation^{1,4}. The conduction band is formed from a linear combination of the singly occupied molecular orbitals (conveniently termed conduction molecular orbitals (CMOs)) on each molecule in the one-dimensional lattice. The systems are usually treated in terms of the Hubbard⁵ Hamiltonian, and within this model the Hartree approximation indicates that the metal-insulator transition^{6,7} will occur at $U_{\text{eff}}/4t = 1$, where U_{eff} is the energy of an ionic fluctuation ($\dots M \dots M \dots \rightarrow \dots M^+ \dots M^- \dots$) and t is the intermolecular transfer integral which arises from overlap between the CMOs on adjacent sites M (where $M = \text{TTF}^+$, TCNQ^- or OAH).

For comparison I choose the ionic charge transfer salt (TTF-TCNQ) (refs 1, 8 and 9), the CMOs of which are shown in Fig. 1 together with those of OAHs phenalenyl (PLY) (refs 3 and 10 and my unpublished work) *sym*-tribenzophenalenyl^{11,12} and *sym*-nonabenzophenalenyl. The various criteria which are thought to be important in the design of organic metals and superconductors have been thoroughly discussed by others^{1,13-15}; they are: (1) The existence of unpaired electrons. Neutral OAHs are free radicals and as such possess one unpaired electron in a non-degenerate MO (just as TTF^+ and TCNQ^-).

(2) A uniform crystal structure. Unlike TTF^+ and TCNQ^- , no counter-ion is required for OAHs which may form totally homogeneous crystals.

(3) Relatively weak electron-electron repulsion (minimisation of U_{eff}). SCF MO calculations (my unpublished results) show that PLY has an ionic fluctuation (or disproportionation) energy of the same order of magnitude as TTF^+ and TCNQ^- . This stems from the favourable dispersal of charge and the absence of di-ions in this process (that is, $2 \text{OAH} \rightarrow \text{OAH}^+ + \text{OAH}^-$; compare with $2 \text{TCNQ}^- \rightarrow \text{TCNQ}^- + \text{TCNQ}^{2-}$) in this latter respect OAHs are more like normal (Group IA and IB) metals. This is particularly dramatic when it is remembered that PLY is a hydrocarbon, and a further reduction in the ionic fluctuation energy would be expected in systems which are appropriately substituted at the active positions, and in the larger OAHs where there is an even greater delocalisation of charge density.

(4) Maximisation of the intermolecular transfer integral (t). This may be accomplished in two basic ways: First, by maximisation of the degree of overlap between the CMOs of adjacent molecules in the stack; second, by reducing the intermolecular spacing. As shown below, OAHs may be expected to prove superior to ionic charge transfer salts on both counts.

The TCNQ^- ions in charge transfer salts are known to occur in a variety of columnar stacking arrangements¹⁶⁻²⁰, but in none of these are the TCNQ^- ions directly over each other, so as to give optimal overlap of the CMOs. In the highly conducting salts, such as (TTF-TCNQ) (ref. 20), the ions stack to give overlap patterns of the CMOs which are essentially translated by one node in the wave-functions, and probably correspond to the second best phase relationship between adjacent molecules. The non-optimal stacking arrangement in charge transfer salts may be attributed to a combination of two factors: the need to maximise the coulomb attraction with counter-ions²¹ (see (2) above), and the unfavourable intracolumnar electrostatic interaction between the high charge densities which are present in these ions. This repulsion is maximised in a completely superimposed stacking arrangement (even in the offset configuration the interaction is high, see below). This contrasts with the case of OAHs which are neutral radicals, and where the local charge densities are extremely small (zero in the HMO approximation). It is therefore to be expected that OAHs will adopt a totally eclipsed stacking arrangement with optimum overlap of the CMOs, particularly if the unpaired electrons in charge transfer compounds make a significant contribution to the cohesive binding of the crystal²¹ (as in

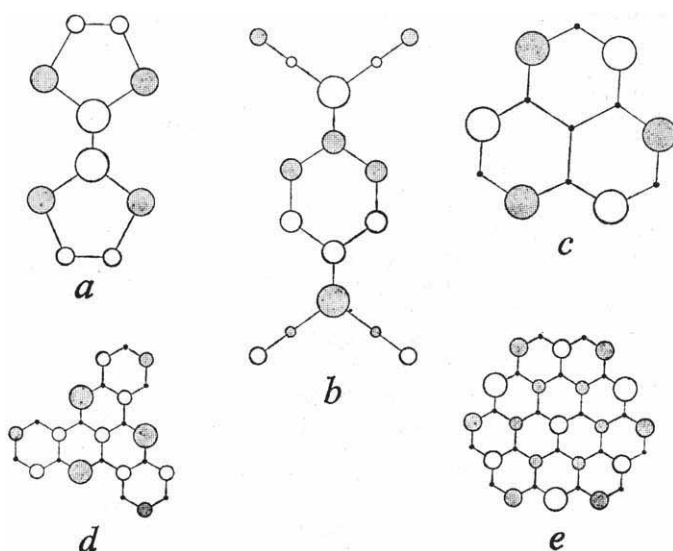


Fig. 1 The CMOs of TTF^+ (a), TCNQ^- (b), PLY (c), *sym*-tribenzophenalenyl (d), and *sym*-nonabenzophenalenyl (e). The CMO is the highest (singly) occupied MO in the molecule and forms the basis for the conduction band in one-dimensional face-to-face charge transfer compounds. The dimensions and wave functions of (a)-(c) were calculated by SCF MO theory, whereas (d) and (e) are shown with HMO wave functions and standard geometries². Open and stippled circles represent the positive and negative coefficient, respectively, of the $2p\pi$ atomic orbital; $\cdot$, $\cdot$, denotes a node in the wave function.

normal metals).

Turning now to the second point, the reduction of the intermolecular spacing in charge transfer compounds, it is appropriate to begin by considering the Madelung constant (α) of a one-dimensional lattice of positive or negative point charges (which are assumed to represent TTF^+ or TCNQ^-). The result is the same in both cases, and the Madelung constant is given by $\alpha = 2(1 + \frac{1}{2} + \frac{1}{3} + \frac{1}{4} + \dots)$. This series is divergent, and as the magnitude of the repulsion between adjacent sites is quite large (a value of 3.0 eV has been estimated²² for TCNQ^- ions), it is apparent that the intermolecular approach between adjacent molecules in salts such as TTF^+ and TCNQ^- is limited by this unfavourable electrostatic contribution to the cohesive binding energy of the crystal along the one-dimensional axis. Of course OAHs, being neutral molecules, have a Madelung constant $\alpha = 0$. With the removal of the coulombic repulsion along the one-dimensional axis, decreased intermolecular spacing would be expected to follow. Furthermore, the intermolecular spacing between adjacent TCNQ^- molecules has been shown to exert a profound effect on the electronic structure of these solids, and it is significant that the salt with the highest conductivity so far^{1,8,9} has the closest approach between adjacent TCNQ^- ions²⁰. This is not surprising as t is of exponential form, and at the characteristic separation shows great sensitivity to distance. For example, a decrease in the intermolecular separation of 0.2 Å from the "normal" value of 3.2 Å (TCNQ^-) leads to an increase in t of more than 30% (my unpublished work).

(5) The question of superconductivity in organic metals is now in a state of flux (see refs 1, 9, 13-15, 23-29). A full discussion of the relevance of OAHs to this general area will be deferred to a later paper; the likely differences in the phonon spectra of OAHs and conventional systems such as TTF and TCNQ suggest that OAHs hold promise in the design of organic superconductors. In the parent OAHs the coupling between the intramolecular vibrational levels and the conduction electrons is minimal (zero in the HMO approximation), as the CMOs are formally non-bonding and the occupancy of these orbitals therefore makes no contribution to the bond order. This contrasts with systems such as TTF and TCNQ, where in the case of TCNQ electron addition progressively

changes the molecular character from quinonoid to benzenoid with accompanying nuclear reorganisation. This effect (which in the crystal may be viewed as the intramolecular phonon contribution to the polaron binding energy) has been implicated in both the high conductivity^{1,25} (as a mechanism for electron-pair formation), and in the non-metallic transition observed for (TTF-TCNQ). In the latter case it has been suggested³⁰⁻³³ that the librational distortion provides the driving force for a Peierls transition³⁴; clearly this behaviour would be inhibited in OAHs where, in the case of PLY, I calculate the distortion energy to be decreased by at least an order of magnitude. Furthermore, if the intramolecular contribution to the transverse optical branch of the phonon spectrum does make a contribution to the high conductivity of salts such (TTF-TCNQ)^{1,25}, an analogous effect may be introduced into OAHs in an easily controllable manner by exocyclic substitution at the active positions. Finally it is appropriate to point out that ionic fluctuations in charge transfer compounds will always be strongly coupled to the longitudinal acoustic branch of the phonon spectrum and in the case of OAHs will lead to a shortening of the intermolecular spacing (with concomitant increase in the transfer integral) due to the formation of an adjacent cation-anion pair, which should be particularly favourable to the collective mode involved in Frohlich-type superconductivity^{23,24,35} and may even stimulate cooperative polaron formation between chains.

Of the OAHs, PLY is probably best known³, and there is an extensive chemistry for cation, neutral radical (there is some tendency towards dimerisation in solution^{3,10}), and anion; nevertheless, one of the chief attractions of OAHs is the diversity of structures and substitution patterns which may be chosen from, in order to satisfy the design specifications of organic metals and superconductors.

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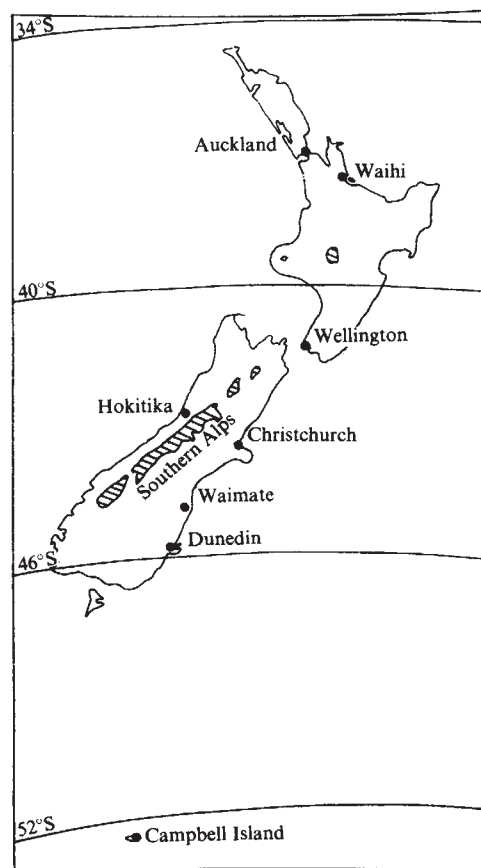
Recent climatic warming around New Zealand

MANY papers have described the climatic fortunes of areas in the Northern Hemisphere over the past few hundred years; the progressive warming during 1890-1940, and the present deterioration. Some people have suggested that the globe could be descending into an ice age (see ref. 1), but such assumptions are derived mainly from data for the Northern Hemisphere. Moreover, data for the Southern Hemisphere used^{2,3} so far have chiefly been obtained from locations between the Equator and 40°S. Information from higher latitudes, where any variations are amplified, is sparse. Here we present the results of an examination of a small area in the mid-latitudes of the Southern Hemisphere which has been warming over the past thirty years.

New Zealand is a long narrow country which straddles the latitudes 34°-47°S, with a SW-NE oriented axial range, the Southern Alps, rising to 3,764 m in the South Island. The southern westerly wind belt which crosses southern New Zealand gives higher precipitation on the windward west coast, and less on the leeward east, whereas the north of the country protrudes into the subtropical high pressure belt.

Extensive records of temperature, rainfall and air pressure for ten locations (Fig. 1) were analysed: six urban, two rural, and two small islands^{4,5}. All stations had minimal site changes except Auckland where temperature correction factors were applied. The data were investigated using 5-yr (short term variation) and 20-yr (longer term trends) running means. The rural stations were used to check that changes seen in urban areas were not attributable to the

Fig. 1 Location diagram of all sites in the New Zealand region quoted in this report. Hatched area indicates land above 2,000 m.



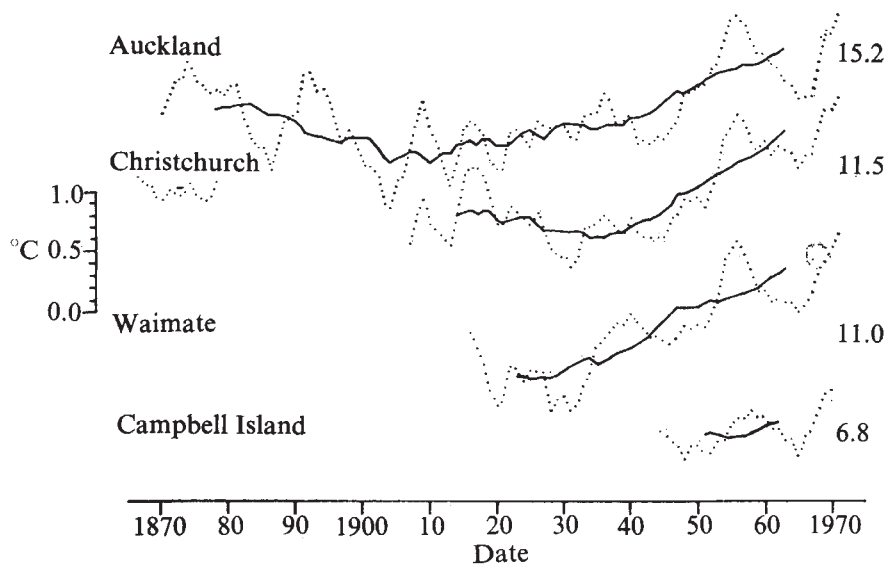


Fig. 2 —, Twenty-year running means of mean annual temperature showing trends typical of New Zealand locations; . . ., five-year running means. Temperature scale shown diagrammatically at left. Numbers indicate average annual mean temperature of the entire station record. Breaks in graphs indicate incomplete records.

heat island effect found in cities. Auckland barometric pressure acted as a position indicator for the subtropical anticyclonic belt. The Auckland-Dunedin pressure differences was used as an index of the strength of the westerlies over New Zealand.

Figure 2 shows typical temperature fluctuations and variations in the New Zealand area. Fluctuations over the whole region are in phase, though of different amplitudes. The urban heat island effect does not interfere with the

now the area is enjoying its warmest spell since temperature measurements began. The warmest single year yet recorded over all New Zealand was 1971.

Rainfall analysis over New Zealand shows no significant temporal variation. Air pressure analysis (Fig. 3) reveals higher pressure over the country around the early 1890s when weak westerlies occurred. The subtropical ridge has migrated north gradually since 1894 accompanied by a great strengthening of the southern westerlies over New

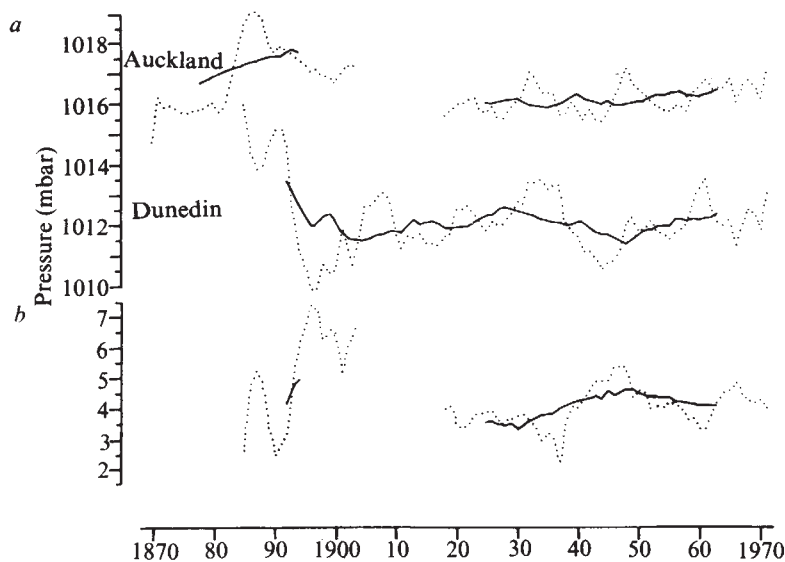


Fig. 3 *a*, Running means of mean annual air pressure at Auckland and Dunedin; *b*, running means of the Auckland-Dunedin air pressure gradient. Notation as in Fig. 2.

trends observed; Waimate, a rural centre 180 km from Christchurch, has variations almost identical to those of urban Christchurch; Waihi and Auckland respond similarly.

The climatic vicissitudes show no great fluctuations in the initial record up to 1880 (Fig. 2). Climatic deterioration occurred in the two decades 1880–1900, the temperatures being cool about 1885, and mild around 1893, but with a declining trend over the whole of New Zealand. The period 1900–1935 was the coldest over the country in recorded history with the years about 1904, 1913, 1921 and 1931 being especially harsh. Climatic amelioration has been experienced throughout the whole region during 1935–1970, temperatures climbing by 1 °C over these years. The mid-1950s were very much warmer than average and

Zealand by 1898, and strong westerlies persisted for the next 10 yr. The migration of the subtropical ridge, with strengthening of the westerlies could well explain the climatic deterioration experienced. Auckland barometric pressure has risen slowly since records began again in 1918 indicating a slow and continual southward shift of the subtropical anticyclonic belt over the past 50 yr. The westerlies over New Zealand weakened around 1935, the time when the present amelioration began. Apart from a small strengthening of the westerlies around 1946 when a small downward fluctuation in the rising temperature trend occurred, the westerlies have not been strong. The southerly migration of the high pressure zone and weaker westerlies over New Zealand seems to explain the recent warming.

Our results reveal that climatic changes in the New Zealand area do not parallel published curves for the globe^{2,3}, so we must re-examine the validity of climatic trends for the Southern Hemisphere computed with little regard to data from points south of the 40th parallel. New Zealand was cooling in the period 1880–1900, and had its coldest period during 1900–1935 when the Northern Hemisphere was experiencing some of the warmest years on record². Conversely, in the past three decades the New Zealand area has warmed up while northern continents have been cooling rapidly. The five year running means of Scott Base, Antarctica, show a rise from -21°C in 1960 to -15.9°C in 1969 (ref. 5). Of eight main Australian urban centres examined from statistical publications for individual Australian states^{6–8}, seven show at least a 0.5°C rise since 1945. Orcadas Island at latitude $60^{\circ}45'\text{S}$ near South America, similarly has warmed¹ by 0.5°C since 1940. So it seems that the warming in the New Zealand region is common to a wider range in the Southern Hemisphere.

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Solid state batteries containing silver sulphonium iodide electrolyte

THE high ionic conductivity α phase of AgI can be stabilised at room temperature by the addition of various inorganic or organic salts (such as Ag_4RbI_5 , ref. 1) to the AgI. These systems have attracted considerable interest as electrolytes in solid state batteries^{2,3}.

We have investigated the properties of electrolytes based on silver iodide with various sulphonium iodides, and we have shown that the solids so prepared are good ionic conductors. Conductivities of pressed pellets were measured as a function of the molar ratio of the components and the composition corresponding to the maximum conductivity was used in subsequent formulations. Examples of the systems studied are $\text{AgI}-(\text{CH}_3)_3\text{SI}$, $\text{AgI}-(\text{CH}_2)_4\text{SCH}_3\text{I}$, $\text{AgI}-\text{O}(\text{C}_2\text{H}_4)_2\text{SCH}_3\text{I}$, $\text{AgI}-(\text{CH}_2)_5\text{SCH}_3\text{I}$ (ref. 4) for which the maximum conductivities fall in the range $3 \times 10^{-3} (\Omega \text{ cm})^{-1}$ to $4 \times 10^{-2} (\Omega \text{ cm})^{-1}$. We are now investigating these and related electrolytes in solid state batteries; we have shown that battery systems can be prepared which have good voltage–time characteristics, good mass efficiency and in which the electrolyte is compatible with the electrode material (thus indicating potentially good shelf life). Such a battery has the characteristics of a commercially viable system.

$\text{Ag}_7(\text{CH}_2)_4\text{SCH}_3\text{I}_8$ electrolyte has been used in a typical battery. The anode is composed of Ag powder, electrolyte and graphite or carbon black. It was found that mass efficiency was increased by using silver powder of low particle size (Johnson Matthey Cypher 88 silver powder, 90% by weight having particle size less than $5 \mu\text{m}$).

The cathode, which in these examples is functioning as a source of iodine, is prepared by reacting a sulphonium iodide with iodine to form a sulphonium polyiodide which is then mixed with electrolyte and graphite. In practice, only $(\text{CH}_2)_4\text{SCH}_3\text{I}_3$ (melting point 89°C) and $(\text{CH}_2)_4\text{SCH}_3\text{I}_5$ (melting point 54°C) of the cheap sulphonium polyiodides are useful because the melting points of the others are too low to be of practical use.

The anode, electrolyte and cathode powders are pressed together into a compacted pellet which forms the battery in the subsequent tests. For reasons of compatibility, the system chosen for the initial tests had the same sulphonium iodide in both the electrolyte and in the cathode.

With this system, using $(\text{CH}_2)_4\text{SCH}_3\text{I}$ as the sulphonium iodide, the electrolyte was unstable in the presence of polyiodides higher than the tri-iodide. The evidence for this was:

(1) The resistances of pressed pellets of mixtures of $\text{Ag}_7(\text{CH}_2)_4\text{SCH}_3\text{I}_8$ + iodine, $\text{Ag}_7(\text{CH}_2)_4\text{SCH}_3\text{I}_8$ + $(\text{CH}_2)_4\text{SCH}_3\text{I}_5$ and $\text{Ag}_7(\text{CH}_2)_4\text{SCH}_3\text{I}_8$ + $(\text{CH}_2)_4\text{SCH}_3\text{I}_3$ were taken at various intervals at 37°C . The change (Fig. 1) in resistance indicated the following reactions:

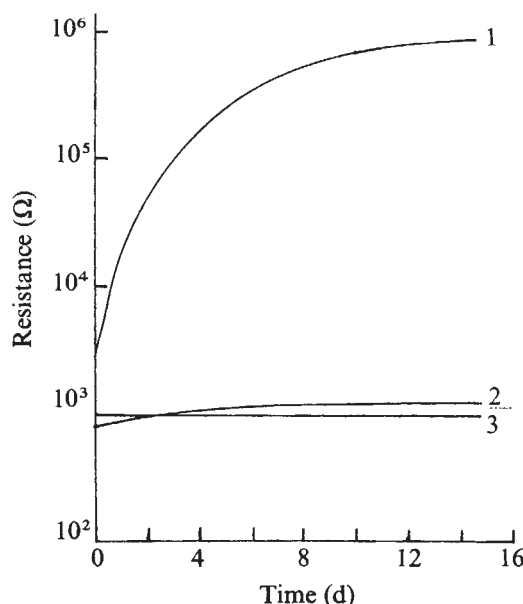
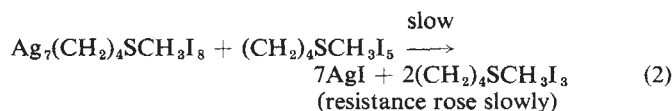
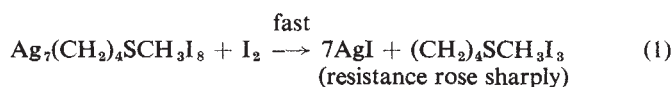


Fig. 1 Time variation of resistance, at 37°C , illustrating results on which equations (1), (2) and (3) are based.

(2) Batteries at 37°C using $(\text{CH}_2)_4\text{SCH}_3\text{I}_3$ in the cathode showed good voltage time characteristics whereas $(\text{CH}_2)_4\text{SCH}_3\text{I}_5$ in the cathode showed poor voltage time characteristics (Fig. 2).

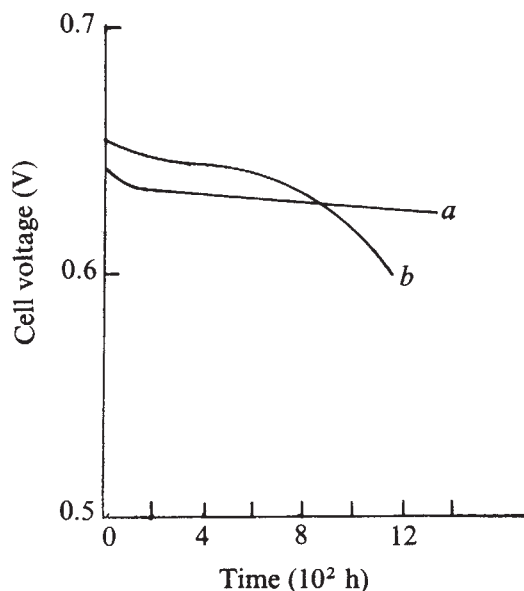


Fig. 2 Time variation of cell voltage, at 37 °C, for cathodes containing: a, $(\text{CH}_3)_4\text{SCH}_3\text{I}_3$; b, $(\text{CH}_2)_4\text{SCH}_3\text{I}_3$.

(3) When the batteries were cut open at the end of the test the electrolyte with $(\text{CH}_2)_4\text{SCH}_3\text{I}_3$ in the cathode still remained a pale yellow colour whereas the electrolyte with $(\text{CH}_3)_4\text{SCH}_3\text{I}_3$ in the cathode had reacted according to the second equation and was much darker in colour.

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Heavy metal binding sites in river water

THE transport of heavy metals (HMs) and their effects on biological systems in natural waters are greatly influenced by their interactions with soluble and insoluble water components^{1,2}.

We have found that ion-specific electrodes can serve as a convenient, precise and sensitive means of measuring the binding ability of solutions for a number of HM ions. We have studied the binding strengths and binding sites of river water for Hg^{2+} , Pb^{2+} , Cd^{2+} , and Cu^{2+} ions, using specific electrodes (see also ref. 3). We used standard membrane filters to retain particles (0.45 μm pore size) as have been used in past studies^{4,5}. We also found that the use of standard ultrafilters to retain macromolecules (in these studies, of molecular weights 45,000, 16,000 and 1,400) may give a much finer definition of the size of binding sites than those obtained previously by filtration studies. These techniques may prove to be very valuable in

studying the dynamics of heavy metal transport and utilisation in natural waters. They could probably be automated for *in situ* determinations of chelation capacity of such waters.

The Hg^{2+} binding capacity (expressed as α , the fraction of the added ion which is bound) of a sample of water from the Ottawa River before and after filtration through different sized ultrafilters is shown in Fig. 1. The ion was strongly bound until 15 p.p.m. had been added; then the bound fraction decreased rapidly, indicating that saturation of the binding capacity of the water had been achieved. Passage of the water through filters, even those retaining molecules with a molecular weight of 1,400 had little or no effect on binding capacity.

The binding capacity of other heavy metal cations showed a different response to filtration. With Pb^{2+} ions, for example, saturation of the metal-binding capacity of water was spread over a much wider range of added HM ions (Fig. 2). Thus, an equilibrium could exist with substantial amounts of free Pb^{2+} ions. Passage of the water through a 0.45 μm filter did not reduce Pb^{2+} binding capacity, but passage through smaller filters significantly reduced it. Some of the Pb^{2+} binding components were less than 0.45 μm in diameter but had molecular weights greater than 45,000. Others had molecular weights between 45,000 and 16,000, and still others had molecular weights of less than 1,400.

The binding capacity of river water for all the ions, and the equilibrium binding constants of these ions with river water are presented in Tables 1–3, which also show the changes in binding capacity for different HM ions caused by the passage through different sized filters. Cu^{2+} ions behaved like Pb^{2+} ions, except that substantial amounts of Cu^{2+} binding activity were removed after passage through a 0.45- μm filter; that is, as shown earlier for other water samples⁴, a good deal of Cu^{2+} binding activity is particulate. The low Cd^{2+} binding ability was unchanged by filtration through any but the smallest filters.

Fig. 1 Binding of Hg^{2+} by river water. α , The bound fraction, is calculated as $(T_M - [\text{M}^{2+}])/T_M$ where T_M is the total HM^{2+} added and $[\text{M}^{2+}]$ is the concentration of free HM^{2+} determined by the specific ion electrode. Increments of $\text{Hg}(\text{NO}_3)_2$ were added to 50-ml samples of Ottawa River water, unfiltered or passed through membrane filters or ultrafilters: a, 45,000 molecular weight filter; b, unfiltered and 0.45- μm filter; c, 16,000 and 1,400 molecular weight filter.

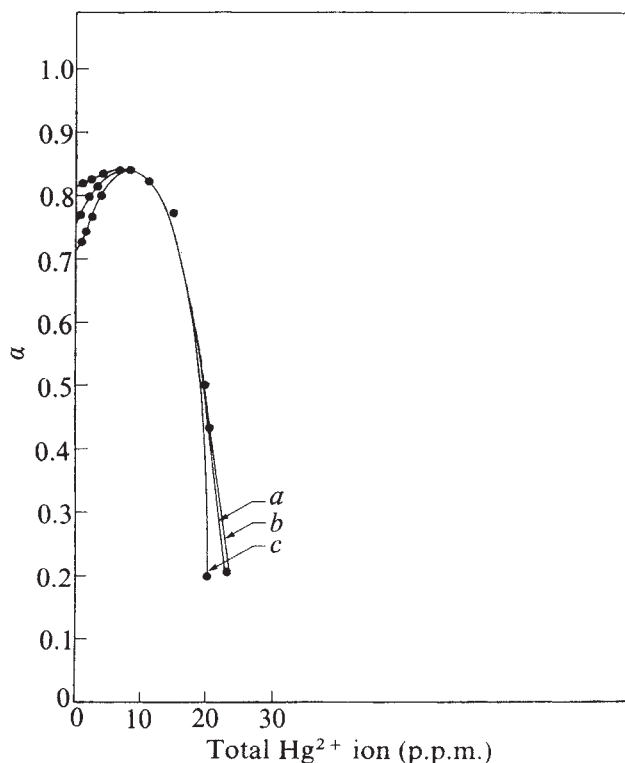


Table 1 Binding properties of Ottawa River water

Conditional constants* for M^{2+} -river water	Equilibrium constants for M^{2+} -fulvic acid complexes	Metal-holding capacity (p.p.m.) of river water	Calculated value for [river component]†
K_{Hg} $1.25 \pm 0.46 \times 10^6$	$Hg^{2+} \approx 5.41 \times 10^{11}$	$Hg^{2+} \approx 11$ p.p.m. at < 15 p.p.m. of $T_{Hg^{2+}}$	5.5×10^{-5} M
K_{Pb} $8.88 \pm 3.8 \times 10^3$	$Pb^{2+} \approx 4.35 \times 10^3$	4 p.p.m. at > 15 p.p.m. of $T_{Hg^{2+}}$	2.0×10^{-5} M
K_{Cu} $5.01 \pm 1.58 \times 10^3$	$Cu^{2+} \approx 2.85 \times 10^3$	$Pb^{2+} \approx 4$ p.p.m.	1.9×10^{-5} M
K_{Cd} $4.69 \pm 0.9 \times 10^3$	$Cd^{2+} \approx 1.81 \times 10^3$	$Cu^{2+} \approx 1.6$ p.p.m.	2.54×10^{-5} M
		$Cd^{2+} \approx 0.6$ p.p.m.	5.4×10^{-6} M
Mean [river component]‡ $2.15 \pm 0.2 \times 10^{-5}$ M			

*Constants calculated from $[M^{2+}]$ measured by ion-specific electrodes.

†Fulvic acid concentration = 10^{-3} M.

‡Calculated as $[M^{2+}]_{bound}$ at equilibrium conditions.

§Mean value calculated excluding value of Hg^{2+} at < 15 p.p.m. $T_{Hg^{2+}}$.

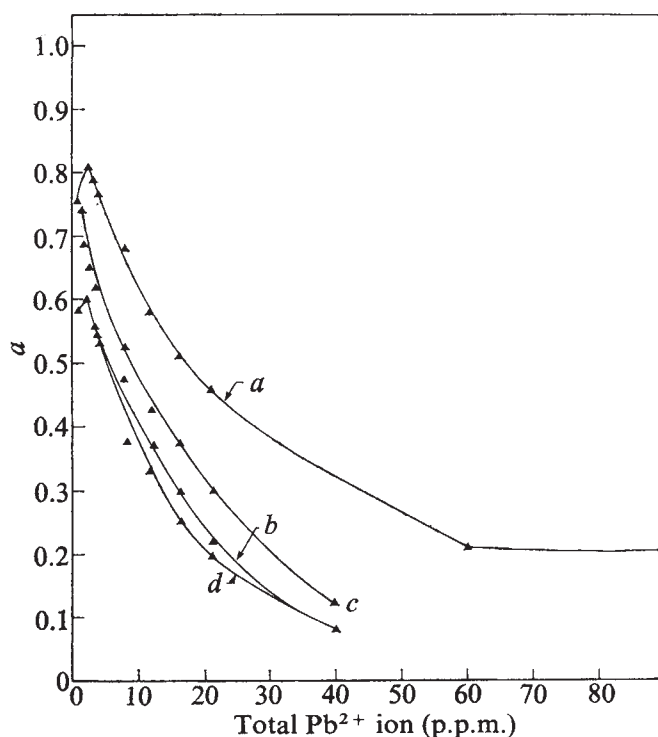
The chemical nature of the HM binding sites in river water is unknown, but we have shown that the binding is not dependent on the presence of HCO_3^- or CO_3^{2-} ions in Ottawa River water. When the water was acidified, gassed with N_2 to drive off CO_2 and neutralised, the binding ability for all HM ions remained the same. In contrast, water from other bodies of water lost a good deal of binding activity after this treatment, implying that HCO_3^- or CO_3^{2-} ions play an important part in such binding. These ions have been implicated in the binding of Cu^{2+} and Cd^{2+} by water from rivers in England^{4,5}. We considered the possibility that other inorganic anions might be involved in HM binding. The level of phosphate in this water (about 1 part per 10^9) was too low to be significant in HM binding. The limit of detection of sulphate was about 5 p.p.m.; if present, such levels could be significant. When samples of ultrafiltered (molecular weight, 1,400) Ottawa River water were evaporated to dryness, ashed (at 900 °C for 16 h) and reconstituted with deionised water, all the HM binding activity was

lost. Separate experiments showed that such treatment did not reduce the HM-binding ability of pure Na_2SO_4 solution. We know of no other inorganic anions likely to be present in significant amounts. The ultrafiltered water is free from suspended particles or macromolecules, and so binding must be a result of the formation of chemical complexes rather than of physical adsorption on surfaces. We think it very probable that the complexing components are organic molecules, destroyed or volatilised during ashing.

Table 2 Total loss (%) in metal-binding capacity after ultrafiltration

M^{2+}	Filter types			
	0.45 μ m	45,000 m.w.	16,000 m.w.	1,400 m.w.
Hg^{2+}	0	0	11.7	11.7
Pb^{2+}	0	31.9	47.9	56.4
Cu^{2+}	34.4	82.6	82.6	82.6
Cd^{2+}	0	0	0	46

Fig. 2 Binding of Pb^{2+} by river water. Calculations as in Fig. 1. $Pb(NO_3)_2$ was added to river water samples. a, Unfiltered and 0.45 μ m filter; b, 16,000 m.w. filter; c, 45,000 m.w. filter; d, 1,400 m.w. filter.

**Table 3** Metal-binding capacity (p.p.m.) of ultrafiltered (molecular weight 1,400) river water before and after removal of inorganic and organic carbon compounds

M^{2+}	Untreated	Inorganic carbon removed	Total carbon removed
Hg^{2+}	11.0	11.04	0.6 p.p.m.
Pb^{2+}	4.0	4.0	No binding
Cu^{2+}	1.6	1.7	0.075 p.p.m.
Cd^{2+}	0.6	0.55	No binding

The possibility that fulvic acid, probably the most important soluble 'humic substance' in natural waters⁶⁻⁸, played a major part in HM binding was investigated by comparing the binding pattern of a sample of fulvic acid isolated from Podzol B_h soil⁸ with the binding pattern of Ottawa River water towards different HM ions. Quite a different pattern was obtained: a 10 p.p.m. solution of fulvic acid had about the same binding ability towards Hg^{2+} ions as did river water, but a much weaker ability to bind the other heavy metal ions than did filtered or unfiltered river water samples. A comparison of the equilibrium constants of Ottawa River water and fulvic acid with the different HM ions (Tables 1-3) shows that fulvic acid cannot account for the HM binding capacity of this water. Studies on the HM binding pattern of fulvic and humic acids isolated from natural waters could help in deciding the role of these complex groups of substances in metal binding and transport by water systems.

A more detailed description of this work and information on the HM binding of other bodies of water has been submitted

for publication elsewhere. The work was supported by the Ottawa River Project, a joint study by the National Research Council of Canada Laboratories and the University of Ottawa.

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Controlled growth of crystalline silicate fibres

DURING studies of the crystallisation kinetics of aluminosilicate glasses of approximately metasilicate composition, surface nucleated crystals have been observed to grow in a fibrous habit¹. This has been attributed to the presence of β -CaSiO₃, the low-temperature polymorph of calcium metasilicate, the mineral equivalent of which, wollastonite, occurs naturally in a similar form.

To produce β -CaSiO₃ fibres in a more controlled fashion it is necessary to increase crystal growth rates in these glasses so that a zone heat treatment can be applied. An investigation by hot stage microscopy of the crystallisation kinetics of various compositions in the CaSiO₃ phase region has revealed that certain ternary glasses possess satisfactory growth-rate/temperature characteristics for this purpose. A glass of composition CaO–SiO₂–ZnO (30:50:20 weight %) was chosen as the most suitable material for initial experiments as it can be drawn easily into vitreous rods of about 1 mm diameter.

Attempts to induce longitudinal crystallisation in these rods by moving them slowly past an annular heating element proved ineffective as not only did crystals nucleate from the surface but the heated zone also slumped considerably, thus

forming a brittle and distorted product. But if the rod is passed through an electrically heated platinum coil supporting a molten zone, any such crystals are removed, and β -CaSiO₃ crystallises from the supercooled melt on the far side in a truly aligned and fibrous habit. Since the glass does not pass through a low temperature before crystallising, neither surface nor internal nucleation can occur spontaneously.

The essential features of this experimental arrangement are shown in Fig. 1, together with an estimated longitudinal temperature profile and the corresponding growth rate profiles. Crystallisation is initiated by introducing a platinum probe into the melt at the start of the process, and thereafter the crystal front is self-stabilising at a position and temperature where its growth rate is equal to the speed of travel of the rod. The temperature of crystallisation in a particular rod thus depends solely on its speed of travel, whereas any variation in heating power to the molten zone (which must be above the liquidus of the glass) merely changes the shape of the temperature profile and therefore the position of the crystal front. Since the glass composition is such that the α/β -CaSiO₃ inversion temperature is situated on the growth rate curve above the temperature peak, speeds below about 20 $\mu\text{m s}^{-1}$ will cause the high-temperature polymorph α -CaSiO₃ to crystallise, whereas speeds greater than the growth rate maximum of about 50 $\mu\text{m s}^{-1}$ will produce a non-crystalline glass rod.

Examination of thin sections of rod by optical microscopy reveals that β -CaSiO₃ crystallises as an array of parallel fibres some 5 μm in diameter right through the rod, so that only a few percent of residual glass remains. Laue X-ray patterns confirm a very high degree of orientation, with the crystallographic *b*-axis (which corresponds to the chain direction of the SiO₄ tetrahedra) aligned longitudinally. There is, in addition, some orientation in the plane perpendicular to the fibre axis, apparent in scanning electron micrographs of fracture surfaces (Fig. 2). In contrast, α -CaSiO₃ grown from the same glass composition but at lower speeds exhibits very little fibrous character or fracture behaviour, and the Laue pattern shows no recognisable orientation of its Si₂O₇ ring structure.

Rods of α -CaSiO₃ which are 1 mm in diameter have a rupture strength of 320 MN m⁻² and an elastic modulus of 91 GN m⁻². These values are virtually identical to those of the base glass; but β -CaSiO₃ rods show significantly higher values of 540 MN m⁻² and 124 GN m⁻², respectively. By comparison, rods prepared by conventional methods, which contain fairly random orientations of surface nucleated and internally nucleated crystals are too delicate to allow three-point bend tests to be carried out.

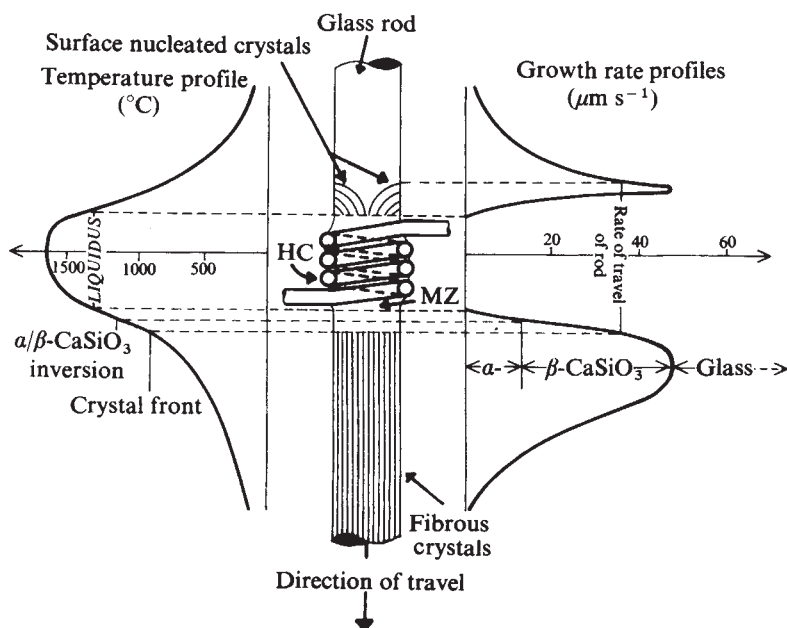


Fig. 1 Schematic diagram of apparatus for crystallisation from the melt (not to scale). The longitudinal temperature distribution, difficult to measure directly, is inferred to be approximately exponential, and corresponding growth rate curves are drawn from hot-stage microscopy data. HC, Heating coil; MZ, molten zone.

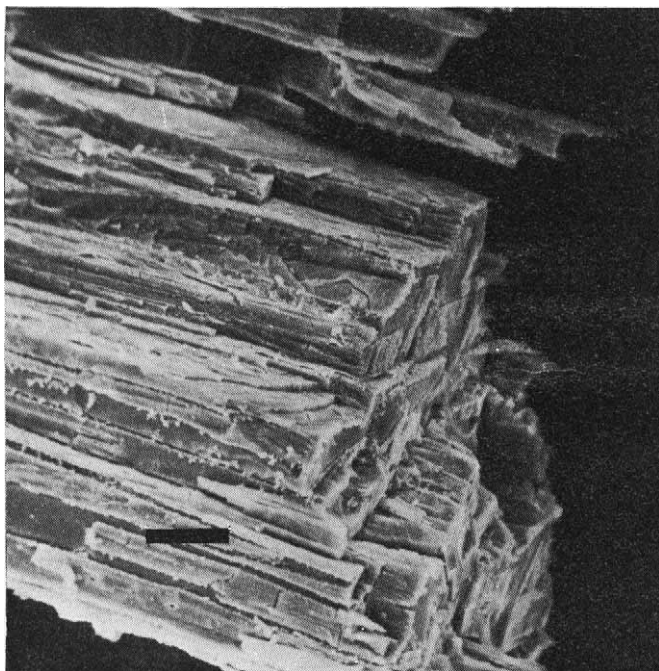


Fig. 2 Scanning electron micrograph of β -CaSiO₃ rod fractured in three-point loading (longitudinal view). Scale bar: 20 μ m.

This novel technique of directional devitrification from a molten zone may be extended both to produce aligned crystal growth in rods with larger cross sections and to draw continuous filaments directly from molten glass compositions which will crystallise at higher rates. Furthermore, it could be applied to any silicate or other system which crystallises in a sheet or chain structure.

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New primitive therian from the early Cretaceous of Mongolia

Therian mammals with tribosphenic molars were probably in existence at the beginning of the Cretaceous¹. This conclusion is based on a single heavily worn lower molar from the Lower Wealden (Neocomian) of England, named *Aegialodon dawsoni*. The molars of the Jurassic therians, (Pantotheria and Symmetrodonta) were capable only of puncturing and shearing. Tribosphenic molars have an additional cusp on the upper molars (protocone) which fits into a basin (talonid) on the matching lower molar. Unfortunately, the single lower molar of *Aegialodon* is extensively worn and slightly damaged. Consequently, it is difficult to discuss with confidence the structure of the earliest known tribosphenic molars, or the inferred structure of the matching upper molars, or the occlusal relationships. In this paper a brief description is given of an early Cretaceous mammalian lower molar from the Aptian of Asia. It is almost identical to that of *Aegialodon*. Because it is exceptionally well preserved and practically unworn, it provides critical information on the structure and function of the very early tribosphenic molars.

The Khovboor beds in Guchin Us somon (county) in the province (aymak) of Arvaykher contain a fauna of early

Cretaceous mammals. Collections from this site (Laboratory of Stratigraphy and Palaeontology, Geological Institute, Academy of Sciences in Ulan Bator) consist of numerous isolated teeth and fragmentary mandibles of triconodonts, multituberculates and therian mammals, as well as the isolated lower molar described here. About seven tons of sediments from the same locality have been washed and screened², and the large collection of mammals obtained is housed in the Palaeontological Institute of the USSR Academy of Sciences in Moscow (currently being investigated by Dr B. A. Trofimov). Four new mammalian genera have already been named but not described³: *Gobioconodon borissiakii* Trofimov, *Gobiodon infinitus* Trofimov, *Prokennalestes kozlovi* Trofimov and *Prozalambdalestes simpsoni* Trofimov. The classification line is: Theria of metatherian–eutherian grade; Family Aegialodontidae; *Kielantherium* gen. nov. Dashzeveg; *Kielantherium gobiensis* sp. nov. Dashzeveg (*Kielantherium* is a monotypic genus, the generic diagnosis is the same as for the type species), (Figs 1 and 2).

Holotype: Right lower molar, possibly M₂—GI (Ulan Bator) No. PST 10–14.

Horizon and type locality: Lower Cretaceous (possibly Aptian), Khovboor, Guchin Us somon, Arvaykher aymak, Mongolian People's Republic.

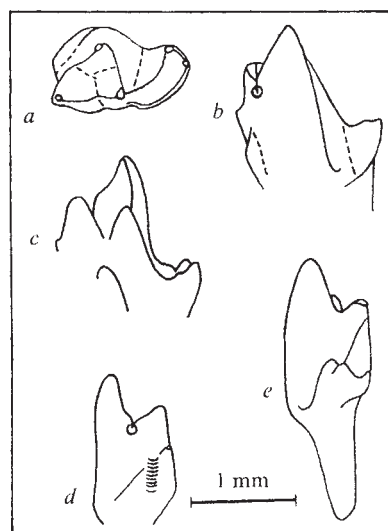
Derivation of name: *Kielantherium*—named in honour of Professor Zofia Kielan-Jaworowska; *therion* Gr., an animal; *gobiensis*, from the Gobi Desert.

Material: The type is a lower molar and is the only representative of this new genus and species in the collection. In the collection in Moscow a mandible with two lower molars, PIN No. 3101–32 seems to be conspecific with the type. The Moscow specimen was not named or cited in the list of species from Khovboor³.

Diagnosis: Lower molar with a relatively narrow talonid, a small hypoconid and hypoconulid and a poorly differentiated ridge supporting two or three crenulations along its medial border, but a distinct ectoconid is not present. The protoconid was considerably higher than paraconid and the latter only slightly higher than the metaconid. Paraconid and metaconid were clearly separated at their bases on the medial face of the tooth.

Kielantherium is assigned to the Aegialodontidae on the basis of a strong similarity to the lower molar of *Aegialodon* in that: the trigonid is small and narrow; shearing surfaces 5 and 3 are small⁴; both anterobuccal and anterolingual cusps are present; relative height of the principal trigonid cusps are the same in both genera; there is a clear separa-

Fig. 1 *Kielantherium gobiensis* gen. et sp. nov. Outline drawings of lower M₂(?). a, Crown; b, exterior; c, interior; d, anterior; e, posterior views.



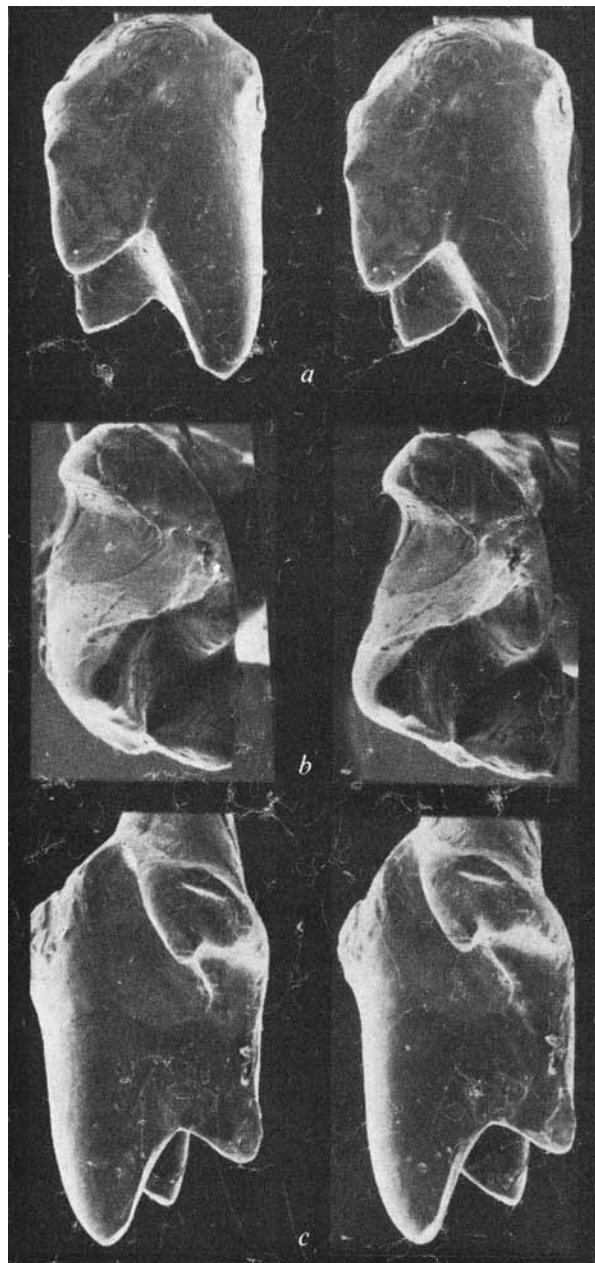


Fig. 2 *Kielantherium gobiensis* gen. et sp. nov. Stereophotographs. a, Posterior; b, crown; c, anterior of lower M_2 (?). $\times 36$.

tion between the bases of the paraconid and metaconid on the medial face of the tooth and; facet 1 can be divided into two subfacets by a line running parallel to the crista obliqua. *Aegialodon* and *Kielantherium* are remarkably similar to *Kermackia texana*⁵ which is based on a single lower molar. The question which obviously arises is whether the type of *Kielantherium* is a premolar rather than a molar. The well developed trigonid cusps, the acute angle between the protocristid and paracristid and the referred specimen with two teeth *in situ* in the lower jaw in the collection from the Khovboor beds housed in Moscow, seem to rule out this possibility.

Kielantherium and *Kermackia* seem to be slightly younger in age than *Aegialodon* and are found in association with more advanced therians; *Prokennalestes* in the case of the former and *Pappotherium* and *Holoclemensia*⁶ in the case of the latter. *Kermackia* and *Kielantherium* therefore seem to be survivors of mammals which had only recently developed a protocone in late Jurassic or early Cretaceous. This suggests that mammals with this feature may have had a wide distribution during the early part of the Cretaceous

(*Kermackia* in North America, *Aegialodon* in Europe, and *Kielantherium* in Asia).

Kielantherium differs from the late Cretaceous Deltheriidae of Mongolia, *Deltatheridium* and *Deltatheroides*⁶⁻⁸, in having a much more primitive structure, with a relatively smaller talonid, smaller talonid basin, in possessing an antero-lingual cusp which is absent in the Deltatheriidae and in smaller dimensions.

I thank Professor A. W. Crompton for reading the manuscript and for helping in its preparation, Dr B. A. Trofimov for permission to study the collection of Khovboor mammals in his charge, and Professor Zofia Kielan-Jaworowska for the loan of the manuscript of her paper.

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Cerebral dominance and reading habits

FREQUENTLY when an Israeli provides travel information in Hebrew, he points in one direction while simultaneously naming its opposite, for example he points to the left while saying: "You must turn right". When this contradictory behaviour is brought to his attention, he excuses himself and either points in the direction he had verbally indicated or corrects his error by changing the verbal expression to correspond with the physical. Observation of such verbal-spatial dissociation prompted the following experiments to explore systematically the possibility that Israelis make more errors in response to questions relating to left-right orientation than do non-Israelis.

Experiment 1 was a screening test. Four hundred right-handed college and high-school students (age range 16-27) participated. Two hundred were native-born Israelis; two hundred were new immigrants to Israel who had arrived within the past three years from Europe and North or South America. Each subject was asked, in random order and in his native language, to respond to a single command: "Look to the left" or "Look to the right." Eye movement responses were recorded by the examiner. Examiners were not informed of the purpose of the test.

Of the 200 new immigrants, 194 (97%) responded immediately and correctly; 6 (3%) responded with hesitation and with an initial movement of the eyes in the wrong direction (4 to the right, 2 to the left), but with subsequent self-correction. By contrast, only 128 (64%) of the 200 Israelis responded immediately and correctly, whereas 72 (36%) responded with hesitation and error (39 to the left; 33 to the right). The difference between native-born Israelis and new immigrants was highly significant ($z > 14.0$, $P < 0.001$; test for z value of significance of difference between proportions). Two types of error were noted among Israelis: 50 subjects (25% of the total Israeli group) responded with hesitation and with initial movement of the eyes in the wrong direction, followed by self-correction; 22 subjects (11%) looked in the wrong direction and did not self-correct. They did correct their direction of gaze, however, when the examiner repeated the command.

This screening test was repeated, in Arabic, with a group of 40 right-handed Arab students (age range 17-32). Twenty-three of the 40 subjects (57.5%) responded immediately and correctly; 17 (42.5%) responded with hesitation and with an initial movement of the eyes in the wrong direction (10 to the left, 7 to the right). Fourteen corrected

their errors without advice from the examiner; 3 did not correct their direction of gaze until the examiner repeated the question.

Experiment 2, a more demanding test of right-left orientation, was presented to 60 right-handed college students. Thirty were native-born Israelis (15 men, 15 women, age range 18-27, mean 21). Thirty were new immigrants to Israel who had arrived within the past three years from various European, North American and South American countries (15 men, 15 women, age range 18-26, mean 22). The test, developed for this experiment, required that the subject respond to an oral command by pointing to parts of the body, either on himself or on the examiner, as the examiner and subject sat facing each other.

Four types of questions were asked. In the 'self-uncrossed' condition, the subject had to point to a part of his own body without crossing the midline (for example, "With your right hand point to your right knee"). In the 'self-crossed' condition, the subject had to point to a part of his own body, across the midline (for example, "With your left hand, point to your right ear"). In the 'examiner-uncrossed' condition, the subject had to point to a part of the examiner's body, without crossing the midline of his own body (for example, "With your right hand, point to my left shoulder"). In the 'examiner-crossed' condition, the subject had to point to a part of the examiner's body by crossing the midline of his own body (for example, "With your right hand, point to my right eye"). Forty commands were given; ten for each condition. Half of the commands involved left hand and half right hand; commands were counterbalanced in each of the four conditions. The forty commands were presented in a predetermined, unpredictable order, and in the native language of each subject by examiners whose mother tongue corresponded with that of the subject. Examiners were not told of the purpose of the study until all testing was completed.

There was a striking difference in performance between native-born Israelis and new immigrants. Of a total number of 1,200 trials per group (30 subjects $\times$ 40 commands), Israelis made a total of 336 errors, whereas new immigrants made a total of 61 errors ($t=13.46$, $P<0.0001$; Student's t test).

Each of the sixty subjects made at least one error. No errors were made in the 'self uncrossed' condition. Most errors were made when subjects had to point to parts of the examiner's body and errors were more frequent in the crossed condition (see Table 1). Significantly more errors were made by the right hand than by the left hand, and by men than by women. These observations were similar for both Israeli and non-Israeli groups. Hesitation before response was more frequent in Israelis (23) than in non-Israelis (11).

These results show that native-born Israelis and Arabs

Table 1 Performance on forty questions of left-right orientation

	Israeli ($n = 30$)	Non-Israeli ($n = 30$)
Errors		
Total	336	61
Examiner crossed	162	30
Examiner uncrossed	127	27
Self crossed	47	4
Self uncrossed	0	0
% of all questions	28	5
Mean per subject (s.d.)	11 (3.5)	2 (1.3)
Range	4-18	1-5
Hand making error		
Right	189	35
Left	147	26
Sex differences		
Errors by males	180	36
Errors by females	156	25

have significantly more difficulty with right-left orientation in response to oral verbal commands than do Europeans and Americans matched for age and educational level. These striking differences may be due to the influence of early-learned reading habits on cerebral function. I propose that reading habits of Europeans and Americans facilitate interhemispheric integration for initiating combined, verbal-spatial activity, whereas reading habits of Israelis and Arabs interfere with this integration.

Israelis and Arabs read from right to left; and Europeans and Americans read from left to right. These different patterns of reading are associated with different patterns of hemispheric activity. When Europeans and Americans start to read a word or line, both hemispheres are activated: the right hemisphere is activated for conjugate deviation of the eyes to the left; the left hemisphere is activated for verbal comprehension. When Israelis and Arabs start to read a word or line, the left hemisphere is activated both for conjugate deviation of the eyes to the right and for verbal comprehension.

Kinsbourne has proposed an attentional model of cerebral organisation which links orientation behaviour (as manifested by direction of gaze) to hemispheric laterality of thought process¹. Thus, when silent verbal problem solving is being carried out, the eyes turn to the right, presumably because of activation of the left (language dominant) hemisphere. When silent visuospatial problem solving is being carried out, the eyes turn to the left, presumably because of activation of the right (spatial dominant) hemisphere. He further suggested that the physiological principle of reciprocal innervation² may apply not only at the spinal cord level but also at the level of the cerebral hemispheres. Accordingly, as one hemisphere is activated in the process of carrying out its functions, the other is inhibited with respect to its own major functions.

Applying these concepts to what was stated above concerning reading habits, we may conclude that initiation of reading for Europeans and Americans involves bilateral cerebral hemispheric activation, and concurrent bilateral hemispheric inhibition. For Israelis and Arabs the onset of reading involves activation of the left hemisphere, with concurrent inhibition of the right hemisphere. When repeated during a period of many years, the act of initiating the reading process gradually fosters the development in Europeans and Americans of bilateral interhemispheric integration for the initiation of tasks which require the simultaneous action of both hemispheres. This is not true, however, for Israelis and Arabs.

Thus, in the current experiments, in which an oral verbal command involving left hemispheric activation required a spatial response involving right hemispheric activation, the initial response of the European and American was more likely to be correct because the two cerebral hemispheres of these individuals had been educated to respond in an integrated manner at the beginning of such combined activity, whereas the initial response of the Israelis and Arabs was more likely to be slower or incorrect, because their brains require more time to allow interhemispheric integration to overcome the effects of the influence of reading habits.

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² Sherrington, C., *Integrative Action of the Nervous System* (Yale University Press, New Haven, 1906).

Electrophoretic differences between sympatric ecotypes

ELECTROPHORESIS has been used extensively for characterising genetic variability within and between natural populations^{1,2} but has not been applied to sympatric ecotypes. In the mosquito *Aedes aegypti* a dark feral form predominates throughout sub-Saharan Africa, but elsewhere in the tropics and moist subtropics a paler domestic form is found. The two ecotypes occur together in Africa in areas with a dry season during which natives store water in pots inside huts^{3,4}. The indoor ecotype breeds continually in water stored in human dwellings, and the outdoor ecotype lives in treeholes and other sites around the dwellings and

The starch-gel electrophoresis methods of Ayala⁵ were used except as noted in the tables.

Gene frequencies for six loci are listed in Tables 1–3. The indoor and outdoor ecotypes differed considerably at three loci. In alkaline phosphatase, the indoor ecotype was fixed whereas outdoor strains had considerable genetic variation. For Coomassie blue (a protein stain), however, the reverse was true—the outdoor ecotype was completely or nearly fixed whereas the indoor ecotype was quite variable genetically. In the outdoor ecotype leucine aminopeptidase bands 0.54 and 0.59 were similar in amount of staining, whereas in the indoor ecotype band 0.59 stains heavily and band 0.54 is usually absent or obliterated by band 0.59. Band 0.59 is nearly always present, and its obliteration of 0.54 made counting frequencies of 0.54 impossible.

Table 1 Gene frequencies for esterase, alkaline phosphatase, Coomassie blue and malic enzyme

Allele position	Esterase			Alkaline phosphatase			Coomassie blue			Malic enzyme		
	0.90	0.86	<i>n</i>	0.69	0.66	<i>n</i>	0.27	0.24	<i>n</i>	1.00	0.88	<i>n</i>
Indoor												
Rabai	1.00	0	82	1.00	0	98	0.16	0.84	89	1.00	0	57
Moyo	1.00	0	98	1.00	0	98	0.29	0.71	87	1.00	0	53
Mgandini*	0.99	0.01	95	1.00	0	95	0.08	0.92	95	1.00	0	90
Outdoor												
Rabai	1.00	0	83	0.75	0.25	101	1.00	0	101	1.00	0	46
Kombeni	1.00	0	97	0.82	0.18	93	1.00	0	87	1.00	0	44
Kombeni*	0.96	0.04	135	0.81	0.19	150	1.00	0	137	1.00	0	152
Kwa Dzivo*	0.94	0.06	99	0.71	0.29	104	0.99	0.01	102	1.00	0	96
Puerto Rico*	0.99	0.01	692	1.00	0	673	0.58	0.42	108	1.00	0	143
<i>A. mascarensis</i>	0.98	0.02	118	1.00†	0	61	1.00	0	130	1.00	0	17
<i>A. albopictus</i>	0.78	0.22	91	1.00	0	76	1.00	0	99	0	1.00	14

The techniques of Ayala⁵ were modified as follows. Esterase, no incubation in borate, 1.5 ml β -naphthyl acetate solution (1:1 water–acetone solvent) added; alkaline phosphatase, stain with no PVP, no NaCl; Coomassie blue, 60 mg Coomassie blue, 90 ml methanol, 18.3 ml glacial acetate, 91.7 ml water, after several hours washed with the same solution to clear background; malic enzyme, 0.056 M Tris-HCl buffer, PMS added only after 1 h incubation. Alleles are assigned positions relative to the distance travelled by the borate band, except for malic enzyme, for which the most frequent allele is assigned position 1.00. There are two esterase alleles at one locus⁷. For alkaline phosphatase, Coomassie blue and malic enzyme, band frequencies do not deviate from Hardy–Weinberg equilibrium assuming two Mendelian alleles at one locus.

*Sampled within one to two generations from field capture. Other samples were from laboratory colonies up to several years old.

†Gene frequency is for pupae only; older larvae have frequencies of 0.39, 0.61 ($n = 28$); half-grown larvae 0.95, 0.05 ($n = 20$).

breeds seasonally. Our data provide the first demonstration of electrophoretic differences between sympatric ecotypes.

The indoor and outdoor ecotypes used all came from an area (several square kilometres) around Rabai, near Mombasa, Kenya—the indoor samples from inside dwellings and the outdoor samples from feral habitats. The Kwa Dzivo sample came from eggs from tin cans nailed to trees in a village. A sample of *A. aegypti* from south-west Puerto Rico (Corozo, Maguayo, Sabana) in the Caribbean, *A. albopictus* from Singapore, and *A. mascarensis* from Mauritius in the Indian Ocean, were studied for comparison.

Crucial to the idea of sympatric polymorphism is the conspecificity of the two ecotypes. Both forms seem to be fully interfertile, with the colour pattern differences subject to continuous variation⁴. In our laboratory interbreeding occurred freely between the two ecotypes (the Mwamsabu indoor and the Bejumwa outdoor strains). Hybridisation experiments were carried out in the laboratory in cages with 20%, 50%, and 80% mixtures of the two ecotypes and 50% of each sex. Promiscuous coupling in *A. aegypti* precludes the use of coupling as a criterion of insemination. We therefore reared the offspring of each female and

Table 2 Gene frequencies of phosphoglucosyltransferase alleles

Allele position	Locus A		Locus B						<i>n</i>
	0.56		0.75	1.00	1.29	1.53	1.76	2.00	
Indoor									
Rabai	0		0.05	0.60	0.35	0	0	0	54
Moyo	0		0.04	0.92	0.03	0	0	0	44
Mgandini*	0		0	0.82	0.13	0.06	0	0	95
Outdoor									
Rabai	0		0	1.00	0	0	0	0	46
Kombeni	0		0.01	0.99	0	0	0	0	44
Kombeni*	0		0.06	0.87	0.07	0	0	0	83
Kwa Dzivo*	0		0.04	0.86	0.10	0.01	0	0	91
Puerto Rico*	0		0	1.00	0	0	0	0	123
<i>A. mascarensis</i>	1.00		0	1.00	0	0	0	0	18
<i>A. albopictus</i>	0		0	0	0.04	0.19	0.58	0.19	13

The four alleles in *A. aegypti* were found by hybridising inbred lines to be at one locus⁸, but band 0.56 of *A. mascarensis* is at a separate locus, and the alleles of *A. albopictus* may be at either or none of the previous loci. The most frequent allele (A1⁵) is designated position 1.00. Techniques are those of Ayala⁵.

*Sampled within one to two generations from field capture. Other samples were from laboratory colonies up to several years old.

Table 3 Gene frequencies for leucine aminopeptidase

Sample	Locus A		Locus B		n
	0.59	0.76	0.81		
<i>A. aegypti</i>	1.00	0	0		1,675
<i>A. mascarensis</i>	1.00	0.98	0.02		61
<i>A. albopictus</i>	0	0	1.00		84

The techniques of Ayala⁵ were modified as follows: incubation in 0.25 M borate; NaOH solution is 0.32 M not 0.2 M. Bands 0.76 and 0.81 are assumed to be two Mendelian alleles at one locus. Bands are assigned positions relative to the distance travelled by borate band. Fifteen bands were observed. In larvae band 0.70 stains heavily and predominates and band 0.66 seldom appears, whereas in pupae both bands, especially 0.66, usually appear and stain equally. Band 0.54 appears only in pupae in *A. albopictus*; it sometimes occurs in larvae of *A. mascarensis* and *A. aegypti*. Pupae of the latter two species sometimes have an additional band (0.36) next to band 0.40.

graded the abdominal scale pattern⁴ to determine the ecotype of the male that inseminated each female. These experiments have so far not demonstrated any significant deviation from randomness in mating within and between ecotypes. Previous authors have always considered them to be one species^{4,8}. We have demonstrated partial reproductive isolation between them; therefore, what we are calling two 'ecotypes' may actually be incipient species, partially isolated by habitat selection.

Persistence of the indoor ecotype in village huts seems to be the result of strong behavioural habitat selection for dwellings⁹. Such habitat selection may enable persistence of fairly discrete gene pools as shown by our data.

We will present elsewhere a mathematical model of the evolution of the indoor ecotype from the outdoor ecotype. We hypothesise that the indoor ecotype arose in sub-Saharan African areas with a dry season, when humans began to store water in pots in dwellings. During the dry season these pots are the only suitable habitat. We believe that habitat selection for the dwellings, and natural selection for different life history attributes and for preference for feeding on man arose during repeated dry seasons. The indoor ecotype has slower larval development, larger eggs, larger adults, and greater resistance to desiccation as adults. The indoor ecotype is preferentially attracted to human odour, the outdoor ecotype to guinea pig odour. Oxygen tension of water must be lower for eggs of the outdoor ecotype to hatch than for eggs of the indoor ecotype. Detailed evidence concerning the ecological differences between ecotypes will be published elsewhere.

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Nitrogen fixation by *Rhizobium* cultured on a defined medium

It has been widely believed that the legume root-nodule bacteria (*Rhizobium* spp.; rhizobia) fix N₂ only within the tissues of the host plant¹. There have however been recent reports that a

strain of cowpea rhizobia, 32H1, developed nitrogenase activity when grown in association with legume or non-legume plant cells^{2,3}. The induction of nitrogenase was apparently due to a diffusible factor(s) secreted by the plant cells, since nitrogenase activity was detected when strain 32H1 was grown adjacent to, but not in contact with, tobacco cells³. Several plant metabolites, including sugars known to favour rhizobial growth^{4,5} and citric acid cycle intermediates^{6,7}, were examined for their effect on this latter system and as possible direct inducers of nitrogenase activity in cultured rhizobia. This led to the formulation of a defined medium on which strain 32H1 fixed N₂ in the absence of plant cells.

This medium (CS7) contained (in mM) KH₂PO₄ (2.2), CaCl₂·2H₂O (0.7), KCl (0.9), MgSO₄·7H₂O (0.14), glutamine (2), myo-inositol (5.6), Na-succinate (25), L-arabinose (25) and (in μM) MnSO₄·4H₂O (58), H₃BO₃ (82), ZnSO₄·7H₂O (3.5), KI (6), CuSO₄·5H₂O (0.8), Na₂MoO₄·2H₂O (0.4), CoCl₂·6H₂O (0.4), FeSO₄·7H₂O (54), Na₂-EDTA (54), thiamine-HCl (15), nicotinic acid (41), pyridoxine-HCl (2.4), and 1% w/v agar (Difco Noble) at pH 5.9. In all experiments the bacteria were initially cultured on yeast-mannitol agar (YMA), suspended in sterile water (10⁸–10⁹ cells ml⁻¹) and spread as 1 cm² areas on CS7 medium. On other plates, similar areas of bacteria were grown 5 mm from uninoculated cell cultures of *Nicotiana tabacum* cv. Wisconsin 38. Cultures were incubated at 29 °C. Segments of agar bearing 1 cm² of dense bacterial growth were excised daily and assayed for nitrogenase activity by the acetylene reduction technique⁸.

Nitrogenase activity was detected on day 4 and increased linearly during the following 4 d (Fig. 1). The rates of C₂H₂ reduction were similar for bacteria cultured in the presence or absence of plant cells. When arabinose (Fig. 1), succinate, or glutamine was omitted from the CS7 medium, nitrogenase activity was low or undetectable. Fumarate was, however, found to be a suitable replacement for succinate and galactose could replace arabinose in the basic medium. The bacteria also showed nitrogenase activity when (NH₄)₂SO₄ (1 mM) replaced glutamine in the medium, but the level of activity was less. The reduction in the glutamine concentration from that used in previous studies of plant cell-rhizobia associations^{3,7,10} followed experiments which showed that 2–10 mM was the optimum concentration range for nitrogenase activity in the tobacco-strain 32H1 association.

The optimal P_{O₂} for C₂H₂ reduction was found to be 0.15–0.20 atm (2.5 and 1.9 nmol C₂H₄ per segment per h

Fig. 1 Time course of acetylene reduction by strain 32H1 cultured on CS7 medium. Agar segments (1 cm²) containing bacteria (about 2 mg bacterial protein⁹ per segment at 8 d) were sealed in vials with serum stoppers, evacuated, and filled with a gas mixture containing 20% O₂: 10% C₂H₂: 70% argon, incubated at 30 °C for 2–3 h and assayed for C₂H₂ reduction by gas chromatography. Activity on CS7 with ● and without ○ arabinose; activity on CS7 plus tobacco cells (see text) with ▲ and without △ arabinose.

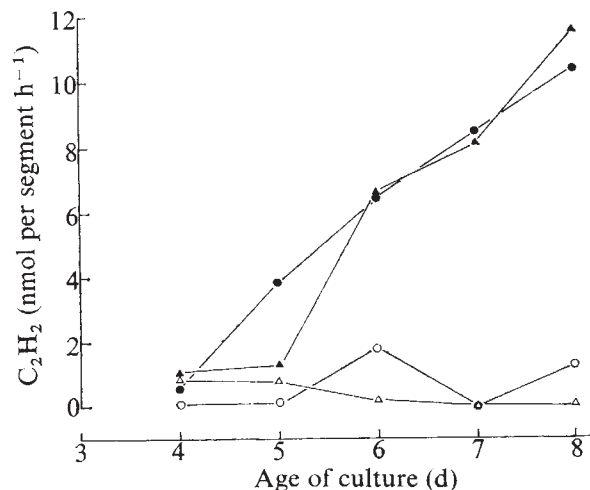


Table 1 Strains* of different *Rhizobium* species assayed for C₂H₂ reduction when cultured on CS7 medium

<i>Rhizobium</i> spp.	Nitrogenase positive†	Nitrogenase negative
<i>Rhizobium</i> sp. (Cowpea group)	CB756, CC751, CC1066, 41F2, 61B9, 127N1, 127N2	CC402, CC430, NU197, 32Z3, 29C2, 8B4, 41A1
<i>R. japonicum</i>	61A76, CB1809, CC705, CC715, CC717, CC713, CC716, CC723	129, CC714, CC718, CC720, CC721 WU274, WU425, CC603, CC624
<i>R. lupini</i>	—	CC8, SU47, SU388, SU574
<i>R. meliloti</i>	—	TA1, WU95, CC2238c, WA67, 2009
<i>R. trifolii</i>	—	TA101, NA505
<i>R. leguminosarum</i>	—	

*The strains, all of which nodulate "Siratro" (including *R. japonicum*) or their homologous hosts, were provided by Dr J. C. Burton, Nitragin Co., Milwaukee, USA, Dr D. A. Phillips, Indiana State University, Terre Haute, USA, Dr J. Dobereiner, IPEACS, Km 47, Campo Grande, Brazil, and J. Brockwell, Division of Plant Industry, CSIRO, Canberra, and we are grateful for their cooperation. Details of strain isolation and source will be provided on request to the authors.

†All positive strains produced >0.5 nmol C₂H₄ per culture per h, with the exception of CC705 (0.2 nmol C₂H₄ per h).

respectively). In assays without O₂ added to the gas mixture (10% C₂H₂, 90% argon), or with P_{O₂} = 0.4 atm, the activity was 0.1 nmol C₂H₄ per segment per h.

Strain 32H1 was cultured on CS7 medium for 7 d and exposed to an atmosphere of 20% O₂ and 80% N₂, containing 80 atoms % ¹⁵N, for 24 h. The bacteria were washed from the agar and ¹⁵N enrichment of the total N of the samples determined⁸. The highly significant values of 0.948 and 0.643 atom % excess for two samples, compared with zero values for control bacteria incubated in air, indicated that the cultures fixed N₂.

In previous experiments, 32H1 was shown to be a pure culture of rhizobia^{2,3}. We have subsequently examined the following clones of strain 32H1 (1) 15 single colonies isolated from YMA cultures³, (2) 12 single colonies isolated from nodules formed following inoculation of cowpea plants with strain 32H1³ (3 isolates from 4 nodules) and (3) 12 single colonies isolated from 32H1 segments on CS7, replicates of which were active in reducing C₂H₂ (Fig. 1). All of these isolates reduced C₂H₂ when cultured on CS7, and also nodulated the tropical legume "Siratro" (*Macroptilium atropurpureum*) grown from surface-sterilised seed on sterile agar slopes in cotton-plugged tubes. No natural C₂H₄ production by strain 32H1 cultured on CS7 was detected, nor did this strain reduce C₂H₂ when grown on YMA medium, or on media³ used previously. Ten single colonies of 32H1 growing on CS7 failed to grow when transferred to nutrient agar (Difco). We therefore concluded that the cultures which fixed N₂ were authentic, pure cultures of cowpea rhizobia.

In addition to strain 32H1, 42 strains from 5 *Rhizobium* species and the cowpea group (*Rhizobium* spp.) were tested for nitrogenase activity on CS7 (Table 1). Of the 6 active strains from the cowpea group, strain CB756, and all 12 single colony isolates from nodules on CB756-inoculated cowpea plants, showed a level of activity comparable to that found with strain 32H1. Three of the strains (61A76, 29C2, 32Z3) tested here were reported previously to form an N₂-fixing association with soybean cells¹¹⁻¹³; of these, only *R. japonicum* 61A76 showed nitrogenase activity on CS7.

These results show that some rhizobia strains can exhibit nitrogenase activity when cultured on a defined medium in the absence of plant cells. This provides direct proof that these rhizobia possess the genetic information for the biosynthesis of the nitrogenase enzyme, thus confirming earlier suggestions of an indirect nature based on genetic¹⁴ and biochemical^{15,16} evidence and results of tissue culture experiments^{2,3}. Significant levels of nitrogenase activity seem to depend on the provision of preferred carbon (for example arabinose, galactose) and nitrogen (for example glutamine, (NH₄)₂SO₄) sources for bacterial growth and a citric acid cycle intermediate (for example succinate, fumarate). The glutamine-synthesising enzymes are important in the regulation of nitrogenase synthesis and ammonia assimilation in *Klebsiella*^{17,18}, and although these enzymes are found in nodule bacteroids^{19,20}, our current results do not permit conclusions regarding a regulatory function for these enzymes in rhizobia. So far, attempts to demonstrate workable levels of nitrogenase activity by strain 32H1 in CS7 liquid culture have been unsuccessful. We suggest

that N₂ fixation by rhizobia may depend on the development of favourable conditions within the mass of bacterial growth.

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Nitrogenase activity in rhizobia in absence of plant host

RHIZOBIA can infect legume roots and fix dinitrogen in the root nodules. Repeated attempts have failed to demonstrate fixation by rhizobia on defined medium or in plant extracts. *Rhizobium japonicum* grown anaerobically with nitrate produces a haemoprotein pattern similar to that of N₂-fixing bacteroids¹ and synthesises the Mo-Fe protein of nitrogenase², but it has seemed that infection of legume cells was a prerequisite for the expression of nitrogenase. It has been shown recently^{3,4}, however, that cowpea *Rhizobium* 32H1 could fix dinitrogen when grown close to, but not in contact with, callus cultures of legumes or non-legumes. This supported other evidence^{5,6} that it was the rhizobia which contained the genetic information for nitrogenase, and showed that its expression was promoted by diffusible factors from plant cells.

To characterise these factors we tested callus cultures of

several plant species, and found carrot and rice most effective. Extracts of carrot cells, or carrot root, when added to LNB5 medium⁷ permitted the expression of nitrogenase (C_2H_2) activity by cowpea *Rhizobium* 32H1 grown in the absence of plant cells. We also screened carbohydrates for their use in promoting the callus-*Rhizobium* association⁷, and found xylose, arabinose and galactose particularly stimulating. Subsequently it was found that when plant callus was omitted, one or the other of these sugars plus sucrose sufficed to induce nitrogenase activity. This communication describes nitrogenase activity in rhizobia growing alone on defined medium.

Rhizobial strains were maintained on a medium containing K_2HPO_4 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; NaCl 0.1 g; yeast extract 0.5 g; $CaCO_3$ 0.5 g; mannitol 10 g; per litre of water. The rhizobia produced effective nodules on their appropriate hosts. Neither growth nor acetylene reduction were observed when the bacteria were subcultured on nitrogen-free media³.

The LNB5 medium⁷ contained KNO_3 1,000 mg; $(NH_4)_2SO_4$ 50 mg; $MgSO_4 \cdot 7H_2O$ 250 mg; $NaH_2PO_4 \cdot H_2O$ 150 mg; $CaCl_2 \cdot 2H_2O$ 150 mg; Fe (as Sequestrene 330) 28 mg; $MnSO_4$ 10 mg; H_3BO_3 3 mg; $ZnSO_4 \cdot 7H_2O$ 2 mg; $Na_2MoO_4 \cdot 2H_2O$ 0.25 mg; $CuSO_4$ 0.025 mg; $CoCl_2 \cdot 6H_2O$ 0.025 mg; KI 0.78 mg; myo-inositol 100 mg; thiamine. HCl 10 mg; nicotinic acid 1 mg; pyridoxine. HCl 1 mg; sucrose 30 g; agar 12 g l^{-1} . The pH was adjusted to 5.5.

Cowpea *Rhizobium* 32H1 reduced acetylene when grown on a defined modified LNB5 medium containing galactose, arabinose or xylose together with sucrose (Table 1). For reasons not clear to us, nitrogenase activity is absent if only one carbon source is available.

A source of fixed nitrogen is necessary for dinitrogen fixation by *Rhizobium* under our *in vitro* conditions. In the case of *R. japonicum* 61A76, there is no dinitrogen fixation when fixed nitrogen is not supplied (Table 2). Fixation occurs in the presence of relatively high levels of ammonia, and is not significantly changed in the presence of L-methionine sulphone, which can interfere with NH_3 repression of nitrogenase. In these respects, *R. japonicum* differs from some other N_2 -fixing organisms such as *Azotobacter vinelandii* and *Klebsiella pneumoniae*⁹. Ammonia can be replaced as N source by 0.002 M glutamine, glutamic acid and aspartic acid. Alanine and leucine are less effective and only slight dinitrogen fixation is obtained with valine or citrulline.

R. leguminosarum TA101 fixes nitrogen on a defined nutrient containing xylose (Table 3). There is scant growth and no fixation in the absence of nitrate. Ammonia apparently neither inhibits nitrogenase nor permits its expression in the absence of nitrate.

Dinitrogen fixation in the presence of fixed nitrogen may seem remarkable, for it is often assumed that nitrogenase is always repressed by fixed nitrogen. Some strains of *Azotobacter vinelandii* will fix dinitrogen in the presence of nitrate, however,

Table 1 Effect of carbon source on nitrogenase activity of cowpea *Rhizobium* 32H1

Carbon source (g l^{-1})		Nitrogenase specific activity nmol C_2H_4 per h per mg protein
Sucrose	20	0
Arabinose	20	0
Sucrose	10	
+mannitol	10	0
Sucrose	10	
+xylose	10	0.47
Sucrose	10	
+arabinose	10	0.82
Sucrose	10	
+galactose	10	0.35

Cowpea *Rhizobium* 32H1 was added to the surface of 10 ml solid medium slant in a 30-ml test tube. The nutrient was the LNB5 medium except that the carbon source was varied as shown in the table. After 6 d, nitrogenase activity and protein content were measured. Each figure is the average of four samples.

Table 2 Effect of ammonia and methionine sulphone on nitrogenase activity of *R. japonicum* 61A76

$(NH_4)_2SO_4$ concentration	Nitrogenase specific activity nmol C_2H_4 per h per mg protein
0.001 M	1.85
0.001 M + MSO	1.89
0.01 M	1.39
0.01 M + MSO	1.16
0.1 M	0
0.1 M + MSO	0
0 + MSO	0
0	0

R. japonicum 61A76 was added to the surface of 10 ml solid medium slant in a 30-ml test tube. The nutrient was LNB5 except that the carbon source was arabinose (10 g l^{-1}) and sucrose (10 g l^{-1}) and nitrate was omitted. Methionine sulphone (MSO) was added at 3 mg ml^{-1} . After 9 d, nitrogenase activity and protein content were measured. Each figure is the average of three samples.

and may use both sources simultaneously¹⁰, and in chemostat cultures of *A. chroococcum*, stable cultures are obtainable in which nitrogenase is only partially repressed by ammonia¹¹. There is no reason to believe that the controls on nitrogenase in rhizobia are exactly the same as those in *Azotobacter* or *Clostridium*, and the symbiotic bacteria may require higher levels of fixed nitrogen to repress the enzyme. It has already been established elsewhere that *R. japonicum* can synthesise the Mo-Fe protein in the presence of ammonia and nitrate².

We only observe acetylene reduction in strains which produce a copious slime. It is probable that this serves to protect the nitrogenase from oxygen¹². The bacteria near the surface of the colony may lower the oxygen tension whereas those below produce nitrogenase. In this case, the specific activities underestimate the activity of the nitrogen-fixing rhizobia.

Table 3 Nitrogenase activity in *R. leguminosarum* TA101

Nitrogen source (mg l^{-1})		nmol C_2H_4 per h per mg protein
KNO_3	$(NH_4)_2SO_4$	
1,000	50	0.91
—	50	0
1,000	—	0.97
—	—	0

R. leguminosarum TA101 was added to the surface of 10 ml solid medium slanted in a 30-ml test tube. The nutrient was LNB5 except that the carbon source was xylose (10 g l^{-1}) and sucrose (20 g l^{-1}). Nitrogenase activity and protein were determined after 14 d.

The significance of our results rests on the assumption that our rhizobial cultures are uncontaminated by nitrogen-fixing bacteria. Absence of *Klebsiella* was demonstrated by lack of growth, acid or gas formation in anaerobic semi-solid peptone media containing mannitol, sucrose, glucose or xylose⁸. Lack of growth on standard nitrogen-free media³ and fixation at pH 5.5 also make it unlikely that our cultures are contaminated by *Azotobacter*. Fixation is not observed if test cultures are maintained under dinitrogen, and this argues against the presence of *Clostridium* or *Bacillus*. We have not detected acetylene reduction in any experiment in which sucrose, glucose, fructose or mannitol was provided as sole carbon source. Fixation was promoted by xylose or arabinose, however. Because pentoses are preferred carbon sources by rhizobia, it is certain that the acetylene reduction activity we observe is due to the non-symbiotic nitrogenase activity by rhizobia.

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Acetylene reduction by *Rhizobium* in pure culture

ASSOCIATIONS between legume callus cultures and *Rhizobium* have been shown to fix nitrogen^{1–6}; we have found that at least one strain of *Rhizobium* will do so when cultured alone on certain media.

The strains of *Rhizobium* used were: 32H1, a cowpea strain (Dr J. Burton, Nitragin Co., Milwaukee, Wisconsin); NGR46, another cowpea strain (Dr M. J. Trinick, CSIRO Wembley, Western Australia); and MU1, an isolate from nodules of *Trema cannabina*, (Mr D. R. Coventry, University of Western Australia). MU1 is a strain able to nodulate siratro (*Macropodium atropurpureum*) and cowpea (*Vigna unguiculata* ssp. *unguiculata*). *Rhizobium* cultures were maintained on a yeast extract–mannitol–potato extract medium⁷.

Confirmation that the initial culture of 32H1 was a *Rhizobium* was obtained by testing it for ability to nodulate cowpea. One series of four pots, each containing five plants, was inoculated with 32H1, another series with NGR46, while a third was left uninoculated as a control. Ten days after the seed inoculation, the appropriate pots were each treated with 100 ml of the test *Rhizobium* suspended in sterile water to give about 10⁸ bacteria ml⁻¹. The controls remained unnodulated, while NGR46 nodulated all plants, as did 32H1. A strain of 32H1 was recovered from the nodules and used in further experiments (it is referred to as the first reisolat).

Cultures from eight individual colonies of the first reisolat were then used to each inoculate pots containing five cowpea plants. Suitable uninoculated controls were again included, and did not form nodules. Twenty-two clones of 32H1 were recovered, at least one from each pot, and these clones are referred to as the second reisolates.

A rabbit antiserum prepared against the first reisolat of 32H1 (ref. 8) showed that the initial strain, the first reisolat and the second reisolates were serologically indistinguishable.

The *Rhizobium* plant symbioses set up^{2,3} used soybean (*Glycine max*) and snake bean (*Vigna unguiculata* ssp. *sesquipedalis*) suspension cell cultures and rhizobial strains 32H1, NGR46 and MU1. *Rhizobium* cultures for inoculation of control bottles were derived from cultures grown on maintenance medium (broth or solid) and were adjusted to contain 1.0 × 10⁷–9.0 × 10⁷ bacteria ml⁻¹ in sterile water. The flat surface of 15 ml of callus medium in a 30 ml McCartney bottle was inoculated with 0.1 ml of rhizobial suspension. The callus culture medium was SCN (ref. 2) with or without 10 mM glutamine and 40 mM succinate⁸. The succinate was added as succinic acid, which lowered the pH of the callus medium to 2.5–3.0; the pH was then returned to 6.0 with KOH before adding the agar. Cultures were grown in diffuse light at 25 °C.

After 14 d, the aluminium caps on the cultures were removed and replaced with a black rubber seal, and an aluminium cap with a hole. Endogenous ethylene was read 24 h later on a Pye-Unicam gas chromatograph fitted with a 1.4 m column of Porapak T and operated at 100 °C. The cultures were then flushed with sterile air in a laminar flow cabinet before the caps were replaced and 0.75 ml of acetylene injected. After 24 h, ethylene was again measured, and acetylene-dependent

ethylene production calculated by subtracting the endogenous production.

Plant calluses with or without rhizobia showed an endogenous ethylene production of 0.08 nmol or less per culture bottle. Rhizobia on callus media had an endogenous ethylene production usually undetectable and never greater than 0.01 nmol.

Soybean callus cultures inoculated with strain 32H1 reduced acetylene at rates comparable to those reported by Phillips^{2,3}, but inoculated callus cultures of snake bean did not do so (Table 1). Strain 32H1 also performs poorly with calluses

Table 1 Acetylene-dependent ethylene production by various rhizobial strains, callus cultures, and associations between them

Rhizobium strain	Plant callus	Mean ethylene production per culture (nmol)	Standard error
None	None	0.08	± 0.02
MU1	None	0.08	± 0.02
NGR46	None	0.10	± 0.02
32H1	None	37.89	± 6.93
None	Snake bean	1.24	± 0.53
None	Soybean	0.03	± 0.02
NGR46	Snake bean	0.09	± 0.06
32H1	Snake bean	1.41	± 0.62
32H1	Soybean	68.39	± 24.98

All cultures on SCN medium supplemented with 10 mM glutamine and 40 mM succinate.

of *Vigna unguiculata*⁸, and varies in performance with different soybean cultivars². Internal variability was often high, as reported by others^{2,4}.

Neither the plant calluses alone nor bacterial strains NGR46 and MU1 showed acetylene reduction. Cultures of 32H1, however, reduced acetylene actively on the SCN medium supplemented with glutamine and succinate.

The first reisolat also reduced acetylene on SCN medium supplemented with glutamine and succinate; neither it nor the initial culture did so on SCN alone, or on SCN supplemented only with glutamine. Twenty-two of the second reisolat clones of 32H1 reduced acetylene on SCN supplemented with glutamine and succinate, but not on SCN alone (Table 2). Since 32H1 associations with callus cultures incorporate ¹⁵N₂ (ref. 5), we assume that acetylene-dependent ethylene production can be attributed to nitrogenase activity.

Contamination by nitrogen-fixing bacteria is an unlikely explanation for our results. It seems improbable that the initial culture and the isolate from the first nodule passage should both have contained a nitrogen-fixing contaminant, and even less probable that eight individual colonies from an apparently pure culture could undergo a second nodule passage and again be reisolated with a nitrogen-fixing contaminant in each of the twenty-two second reisolates. We conclude, therefore, that 32H1 is a *Rhizobium* strain capable of acetylene reduction in these conditions.

Table 2 Acetylene-dependent ethylene production by *Rhizobium* strain 32H1 after second nodule passage

First isolate colony no.	Mean ethylene production (nmol) on		Standard error
	SCN	SCN + glutamine + succinate	
1	0	20.53	± 5.33
2	0	25.97	± 17.87
3	0	20.34	± 4.80
4	0	18.44	± 7.93
5	0	55.10	± 15.02
6	0	54.68	± 13.47
7	0	20.95	± 7.64
8	0	34.71	± 6.68
Mean (77 observations)	0	34.04	± 4.67

The medium used was SCN, or SCN supplemented with 10 mM glutamine and 40 mM succinate.

Table 3 Acetylene-dependent ethylene production by the first reisolate of *Rhizobium* strain 32H1 on SCN medium with various supplements

Additions	Mean ethylene production (nmol)	Standard error
None	0.00	± 0.00
10 mM glutamine	3.17	± 1.42
40 mM succinic acid	0.00	± 0.00
10 mM glutamine+40 mM succinic acid	31.69	± 6.64
10 mM glutamic acid	21.64	± 5.45
10 mM glutamic acid+40 mM succinic acid	40.59	± 10.58
40 mM Na succinate	0.00	± 0.00
10 mM glutamine+40 mM Na succinate	11.16	± 2.39
10 mM asparagine	0.79	± 0.04
10 mM asparagine+40 mM succinic acid	25.79	± 6.74

All amino acids are the L-isomer.

We examined the effects of various media supplements on acetylene reduction by 32H1 (Table 3). Glutamine and asparagine were equivalent in the presence of succinic acid, but glutamine in the presence of sodium succinate was significantly inferior. The acidity of succinic acid was apparently important, but only where an amide nitrogen compound was used. Since neither succinic acid nor sodium succinate alone was effective, an available nitrogen source was apparently necessary. The significant increase in acetylene reduction when succinic acid was added with glutamate (which is acid stable) suggests that an available carbon source was also needed. Since slow-growing rhizobia do not usually grow on disaccharides⁹, the sucrose in the callus medium would be ineffective.

Given that glutamine and asparagine are not available for growth of 32H1, and that relatively slight hydrolysis of them to available glutamate, aspartate or ammonia occurs when they are treated with succinic acid, it seems probable that the responses can be explained by growth requiring available sources of carbon and nitrogen.

Acetylene reduction occurs with 32H1 in association with many types of callus, including non-legumes^{2,3,5,6}, and when callus and rhizobia are not in physical contact⁵, suggesting that the calluses provide substances for growth or nitrogenase production. Snake bean callus seemed to prevent nitrogenase production, since the medium contained the necessary supplements for 32H1 alone (Table 1). This conclusion must remain tentative, since rhizobial numbers in the callus and agar surface systems are not comparable.

The finding that strain 32H1 reduces acetylene on laboratory media directly establishes that genes for nitrogenase are present in *Rhizobium*, as suggested by other workers^{10,11} and renders invalid the theory¹² that part of the nitrogenase in legume nodules might be specified by plant DNA.

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High frequencies of haploid production in wheat (*Triticum aestivum*) by chromosome elimination

THE production of haploid plants from hybrids, followed by chromosome doubling, provides the plant breeder with a means of accelerating the development of desirable true breeding lines. This could be advantageous in breeding programmes particularly among some of the cereals where many generations, taking many years, may be required before an acceptable pure line variety can be obtained.

A major limitation to haploid breeding is the lack of techniques for obtaining haploids in sufficiently large numbers. In cultivated barley (*Hordeum vulgare* $2n=14$) the discovery¹ of a high frequency of "vulgar" haploids in hybridisations with *Hordeum bulbosum* ($2n=14$) by means of chromosome elimination, combined with recent improvements in cultural techniques²⁻⁴, promises to overcome the limitation in this cereal.

So far the possibility that chromosome elimination giving rise to haploids may be exploited in other crop plants has not been investigated. To determine if elimination can occur in hexaploid wheat, *Triticum aestivum* ($2n=6x=42$), crosses were made between the wheat variety Chinese Spring and both diploid and tetraploid *H. bulbosum* as pollen parents.

Seed set was obtained in both crosses (Table 1) although

Table 1 Seed set induced from intergeneric crosses between *T. aestivum* var. Chinese Spring and *H. bulbosum*

Male parent	No. florets pollinated	No. of seeds set	Seeds set (%)
2x <i>H. bulbosum</i>	622	85	13.7
4x <i>H. bulbosum</i>	435	188	43.2

the frequency was much higher using tetraploid *H. bulbosum*. The initial development of the seeds was vigorous but after 14-18 d the seeds showed signs of abortion, the endosperm having degenerated, making embryo culture necessary. The embryos were dissected out in a sterile cabinet, transferred to orchid agar (27-29 g l⁻¹) and placed in the dark at 20 °C until they germinated.

Of the 70 plants obtained (Table 2), 57 were examined cytologically in root tips stained by the Feulgen procedure and, whether produced from crosses with diploid or tetraploid *H. bulbosum*, all were found to have 21 chromosomes indistinguishable karyotypically from the haploid complement of wheat (Fig. 1). Nor did any of the plants as seedlings, or the ten plants which have reached maturity so far, exhibit any of the morphological features of *H. bulbosum*.

The frequency with which haploids were obtained when

Table 2 Plants obtained from intergeneric crosses between *T. aestivum* var. Chinese Spring and *H. bulbosum* using embryo culture

Male parent	No. of embryos cultured	No. of plants produced	Embryos giving plants (%)
2x <i>H. bulbosum</i>	39	11	28.2
4x <i>H. bulbosum</i>	110	59	53.6

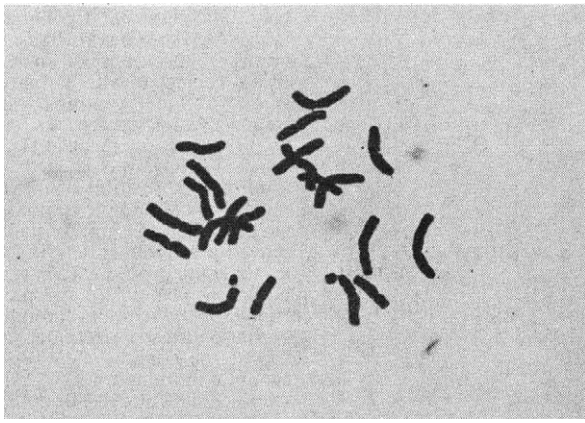
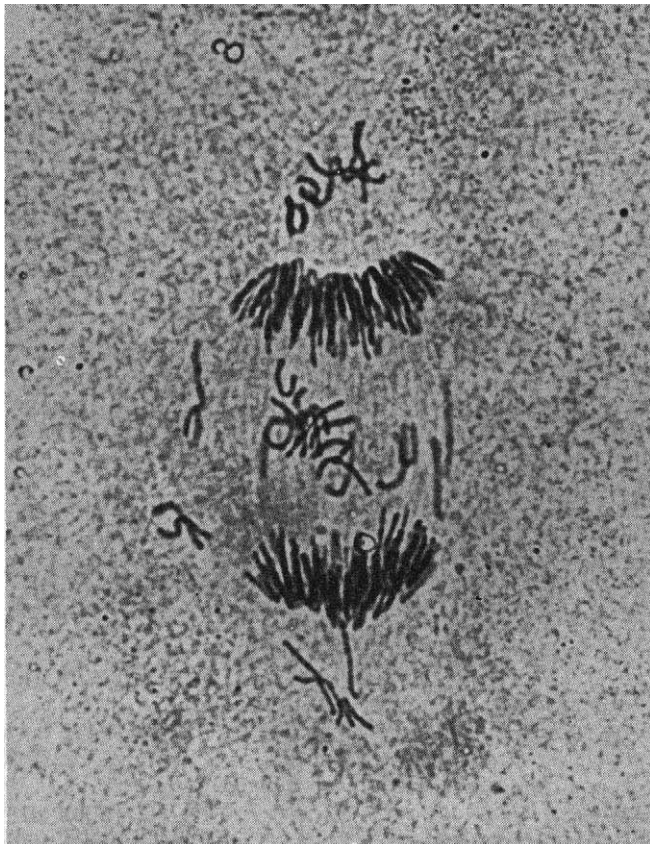


Fig. 1 Somatic chromosomes of a haploid wheat plant ($2n = 21$) obtained by crossing *Triticum aestivum* var. Chinese Spring with diploid *Hordeum bulbosum*. The satellited chromosomes 1B and 6B of wheat are clearly visible. $\times 750$

using tetraploid *H. bulbosum* (13.6% of florets pollinated) is similar to the frequencies being achieved in haploid barley production. The mechanism of production of these haploids also appears to be the same as that for haploid barley from crosses of *H. vulgare* with *H. bulbosum* in which elimination of the *H. bulbosum* chromosomes takes place following fertilisation. In developing seeds fixed 1–6 d after pollination with tetraploid *H. bulbosum*, chromosome counts of 35 at zygotic metaphase indicated that fertilisation was normal. At a later stage, however, the presence of micronuclei in both embryo and endosperm tissues as well as lagging chromosomes and bridges in endosperm divisions (Fig. 2) showed the subsequent elimination of chromosomes.

Fig. 2 Chromosome elimination in a dividing endosperm cell of a developing grain obtained by crossing *Triticum aestivum* var. Chinese Spring with tetraploid *Hordeum bulbosum*. $\times 700$



Investigations are now being carried out to determine whether *H. bulbosum* can be crossed readily with other wheat varieties. These initial results, however, demonstrate the potential of producing haploid wheats by means of chromosome elimination following hybridisation with *H. bulbosum*.

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Visual spatial summation in two classes of geniculate cells

VISUAL perception can be viewed as a transformation and a distortion of the spatial patterns which exist in the outside world. The transformation of external objects into neural images is accomplished by the summation of excitatory and inhibitory influences over the visual field of each neurone. Distortion occurs when the neural response is not simply proportional to the sum of excitation and inhibition because of nonlinear mechanisms in spatial summation. If the responses of all visual cells were distorted by such nonlinear summation, fine visual discriminations like those necessary for reading *Nature* would be impossible. Nonlinear mechanisms do however, seem to be important in one class of visual neurone, perhaps for signalling change or motion in the external world. We have attempted to understand the importance of the different kinds of spatial summation by studying single cells in the cat visual system.

There are several classes of ganglion cells in the cat retina. Two main groups of these cells send axons to the dorsal lateral geniculate nucleus (LGN). These were originally classified and non-committally named X and Y cells by Enroth-Cugell and Robson¹. They found that the main distinction between X and Y ganglion cells is the degree of linearity of spatial summation. A stationary spatial grating, amplitude-modulated in time can be located over the receptive field of an X cell so as to produce no modulation of the impulse rate of the cell. This position of the grating is called the null position. For even a slight relocation of the pattern away from the null position, modulation of the pattern produces modulation of the impulse rate. For Y ganglion cells no null position exists. Signals generated by a change in illumination over one region of the field of a Y cell can never be completely cancelled by signals due to an equal but opposite change in illumination in another region and therefore the Y cells must have some nonlinearity preceding, or at the level of, spatial summation. We have extended this work on the linearity of spatial summation to cat LGN cells. Geniculate cells can also be classified as X or Y on the basis of the linearity or nonlinearity of spatial summation. So X and Y cells are segregated at least as far as the input to the visual cortex. Other workers have used different criteria for grouping retinal ganglion and lateral geniculate cells^{2–4}. These criteria include: axonal conduction velocity, preferential target speed, pattern of response to a drifting grating and response dynamics. We have not investigated the first two of these, but have measured the last two. Elevation of the mean impulse rate in response to a drifting grating is invariably present in Y cells, and we think this characteristic is caused by the same nonlinearity in spatial summation which prevents the cell from having a null position. There is, however, no direct relationship between spatial summation and response dynamics. There are some “transient” X cells and “sustained” Y cells. Because the spatial summation properties of X and Y cells are so distinct, there must be func-

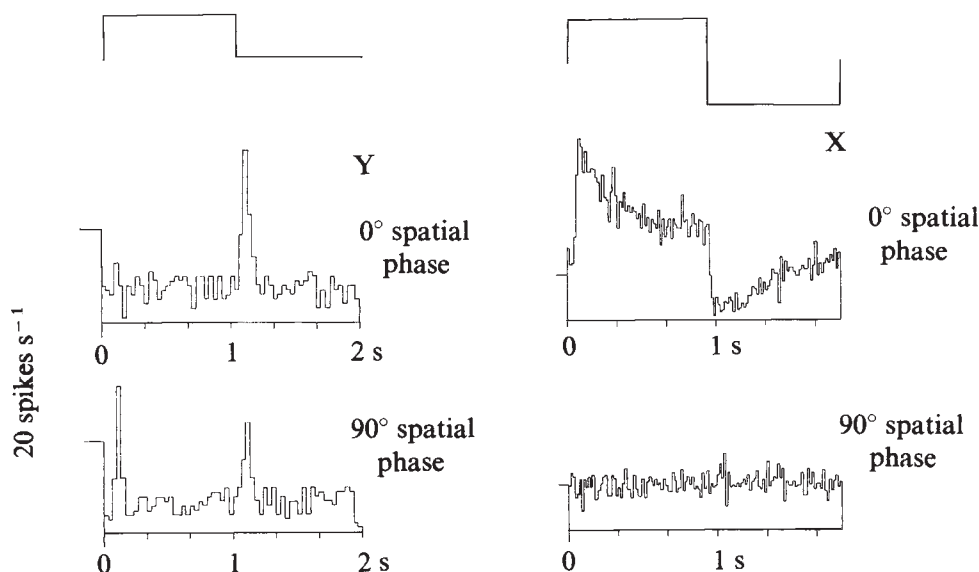


Fig. 2 Contrast sensitivity against position (spatial phase). *a*, An X geniculate cell; *b*, a Y geniculate cell. Sensitivity to elicit an average peak response of 20 impulses s^{-1} is plotted against position of the grating on the abscissa. Zero phase was set by the experimenter to give approximately maximum sensitivity. The ordinate, contrast sensitivity, is the reciprocal of the contrast required to give criterion response. The sign of the sensitivity was determined from the temporal phase of the response. Response at pattern on was considered positive; response at pattern off negative. A sine curve is fitted to the sensitivity function of the X cell. X cell 30/1 on-centre contralateral. Y cell 33/11 off-centre contralateral. Both recorded at LGN. The temporal frequency in both cases was 0.5 Hz; the spatial frequencies were 0.9 cycles per degree for the X cell and 0.45 cycles per degree for the Y cell.

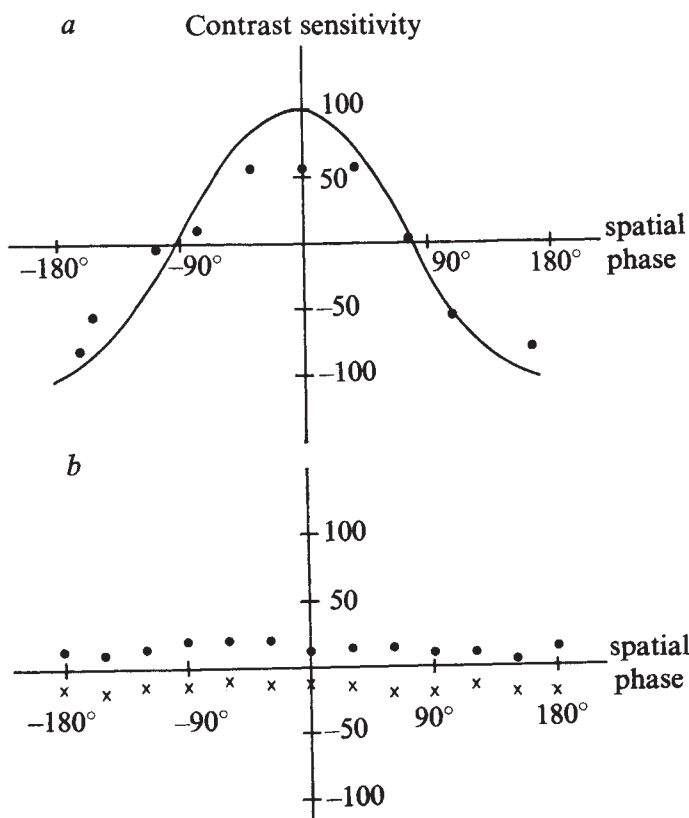


Fig. 1 Null positions in geniculate cells. The right hand column contains two responses (PST histograms of 30 sweeps) of an X geniculate cell to an alternating phase spatial sine grating, spatial frequency 1.8 cycles per degree, temporal frequency 0.54 Hz. The upper response was elicited by the grating at a position of maximum sensitivity. The lower response was elicited when the grating had been moved a quarter cycle (90°) away, to the null position. Contrast for both runs was 2.5%, and the time course of the temporal modulation of the contrast is indicated at the top of the column. Cell 12/1, on-centre, contralateral, LGN afferent recorded at cortex. The left hand column shows data from a Y geniculate cell in response to a 0.45 cycle per degree grating modulated on-off at 0.5 Hz. Contrast for both runs was 10%. Higher spatial frequencies were much less effective in exciting the cell, and response to on-off or alternating phase gratings were very similar when stimuli were equated for contrast. This cell had no null and at certain positions gave a burst of spikes when the grating went on and also when it was withdrawn. Cell 32/3, on-centre contralateral, recorded at LGN.

tional consequences of their parallel input to the visual cortex⁵.

We used 18 X-like cells and 6 Y-like cells in 9 cats anaesthetised with urethane and paralysed with gallamine triethiodide. Action potentials were recorded from LGN cells and from LGN afferents to the striate cortex with tungsten-in-glass micro-electrodes. LGN cells were distinguished from optic tract terminals by impulse wave form and polarity. Nerve impulses were averaged into post-stimulus time (PST) histograms with a computer. The cat looked at an oscilloscope screen through a contact lens with a 3-mm diameter artificial pupil. Refraction was determined with an ophthalmoscope and corrective lenses used when required.

Spatiotemporal patterns were created by means of a television-type raster display, with electronic modulation of the intensity on the oscilloscope screen. The patterns used for the experiments reported here were: (1) an on-off spatial sine grating introduced at a fixed contrast and then blanked, repetitively; (2) an alternating phase spatial sine grating, that is a grating which reversed contrast, or in other words shifted phase by a half cycle (180°), repetitively. For both kinds of patterns, mean luminance across the screen was always constant; only the spatial distribution of luminance varied with time. Pattern contrast was controlled by the experimenter. The mean luminance on the screen was 1 cd m^{-2} .

Some LGN cells had null positions when presented with on-off or alternating phase patterns. These cells are analogous to X retinal ganglion cells. An example is shown in Fig. 1. Shown on the right are the averaged responses of an X-type cell to an alternating phase grating when the grating was at the null position and when the grating was placed a quarter cycle (90° in spatial phase) away from the null. We also found Y-type cells in the cat LGN which had no null position for a grating regardless of spatial frequency and which at some positions produced bursts of impulses at pattern on and pattern off, or at both phases of pattern alternation, as shown for example in the left hand column of Fig. 1 (ref. 1).

We developed a quantitative measure of the strength of the null position of an LGN cell. This was the contrast sensitivity for a grating as a function of position. The reciprocal of the modulation depth required to elicit a criterion peak response, the contrast sensitivity, was plotted against position of the grating. (We called sensitivity for an on response positive and for an off response negative.) Such graphs for an X cell and a

Y cell are shown in Fig. 2. In this case, contrast sensitivity for the X cell at the null was more than a factor of thirty down from the peak sensitivity. For X cells, sensitivity was approximately a sinusoidal function of position of the grating. With linear spatial summation, a visual cell acts like a linear spatial filter for spatial patterns. Among other things, this means that the response to a sine grating must be a sinusoidal function of position, or spatial phase, of the grating in the visual field. The sensitivity profile of the cell need not be symmetrical to yield this result.

There was less variation of contrast sensitivity with position for Y cells, particularly at high spatial frequencies. Also, since at many positions a Y cell responds at both pattern on and pattern off, there are positive and negative contrast sensitivities at these points. Thus, two features of the graph of contrast sensitivity against position served to help classify a cell as either an X or Y cell: sinusoidal sensitivity curve with two nulls or a flatter sensitivity curve and the presence of single or double sensitivity values.

We found LGN cells which had null positions but very transient responses to standing contrast. Cell 30/1 in Fig. 2 was such a cell. Other X cells had a sustained component in their response, for example cell 12/1 in Fig. 1. Y cells with a sustained response also exist. Such results imply that the X/Y classification¹ and the "sustained/transient" classification of Cleland *et al.*^{2,3} (see also Hoffman *et al.*⁴) may not be identical, at least for LGN cells. The classification of visual cells into two groups, X and Y, based on their spatial interactions is as clear and unambiguous, in the LGN as in the retina. Our work shows that the X/Y retinal dichotomy, characterised by the degree of linearity of spatial summation, is preserved all the way to the visual cortex.

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Sporulation-inducing factor in slime mould *Physarum polycephalum*

THE highly synchronous sporulation of *Physarum polycephalum* is an attractive model of cell differentiation. Virtually all of the nuclei in a starved plasmodium proceed in step through the sporulation process in response to a well defined stimulus, light¹⁻⁴. The regulatory mechanisms of this differentiation are unknown but several studies have been presented on the conditions necessary for sporulation. These have been reviewed by Sauer⁵, who concludes that two elements are necessary for sporulation, substrate and light. These must be applied sequentially. The substrate requirement is for starvation in the dark for at least 4 d in the presence of niacin and niacinamide⁴. The light requirement is for at least 4 h illumination by a 40 W fluorescent light. Sauer also cites unpublished results which suggest that the effect of illumination is a local one which cannot be transferred to an unilluminated, starved plasmo-

Table 1 Induction of sporulation by microinjection

Injected substance	Sporulation frequency No. of plasmodia sporulating No. tested	(%)
None	0/4	0
Distilled water	0/10	0
Unilluminated plasmodial contents	0/14	0
Illuminated plasmodial contents	11/18	61
UM10 filtrate	3/5	60
UM10-retained	7/10	70
NaCl (0.06 M)	33/113	29
KCl (0.06 M)	4/33	12
Na ₂ SO ₄ (0.03 M)	2/19	11
(NH ₄) ₂ SO ₄ (0.03 M)	2/16	13

Results are the means of several experiments. Macroplasmodia were established in 9-cm Petri dishes containing sporulation medium (1) in 2% agar. After starvation on this medium for 6 d in the dark at 21 °C, these macroplasmodia were microinjected with 10 µl of the indicated substances and immediately placed back in the dark at 21 °C. Illuminated macroplasmodia were prepared in the same manner except that, at the end of the starvation period, they were placed 6 cm from a 40 W fluorescent light bulb for 6 h. A fan kept the temperature of the dishes below 24 °C. Clarified extracts were prepared by homogenising ten macroplasmodia in 1.5 ml deionised water and centrifuging for 10 min at 26,000g in a Beckman J21 centrifuge. Extracts were then ultrafiltered with Amicon UM10 membranes.

dium or even from the illuminated half to the unilluminated half of the same plasmodium.

In this report we present results which show that the sporulation-inducing effects of illumination can indeed be transferred to an unilluminated, starved plasmodium by microinjection. Furthermore, the requirement for light can be circumvented altogether by the injection of a small amount of salt solution into a starved plasmodium.

The top part of Table 1 shows that a small quantity of plasmodial contents from an illuminated, starved plasmodium will induce sporulation in a starved, unilluminated plasmodium. The sporulation thus induced is usually complete 20 h after injection, but it sometimes takes up to 48 h. Thus, this process is slower than sporulation induced by light, which takes 13-15 h, but it is homogeneous, involving the entire plasmodium synchronously and is morphologically indistinguishable from that observed in an illuminated slime mould. This effect is not produced by injecting an equivalent amount of water, pricking with a micropipette, placing the plasmodium on the dissecting microscope stage in the light for 5 min (the duration of the injection process) or by injecting an equivalent amount of plasmodial contents from an unilluminated, starved plasmodium.

The approximate size of the inducing factor has been determined by ultrafiltration with Amicon membranes. The middle part of Table 1 shows that a factor passes through a UM10 filter and therefore has a molecular weight of less than 10,000. The active substance in the UM10-retained fraction could be the same as that which passes through the filter, as filtration was not taken to completion. It is also possible that there is another, larger component present in the retained fraction.

The bottom part of Table 1 shows that a variety of salts, injected into a starved, unilluminated plasmodium also induced sporulation. The concentration of the cation was held constant in each case. The effect of NaCl seems most pronounced, although the differences may not be significant.

We are unable at this time to determine whether illumination and salt operate through the same or distinct mechanisms of sporulation control. It is evident, however, that there is some differentiation-inducing factor in illuminated slime mould which can be transferred to an unilluminated slime mould. Elucidation of the nature of the triggering

substance(s) may thus help to explain the mechanism of control of differentiation in this organism.

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Inhibition of fungal growth by wheat germ agglutinin

GROWTH of fungal hyphae is the result of a complex and poorly understood process of cell wall synthesis and extension, that is restricted to the hyphal apex¹⁻³. In fungi with chitin-glucan hyphal walls, such as the Deuteromycetes *Trichoderma viride* and *Fusarium solani*¹, hyphal extension and septa formation involve the synthesis of chitin in hyphal tips and septa⁴. We therefore studied the effect on such fungi of wheat germ agglutinin (WGA), a lectin which interacts specifically with chitin oligomers^{5,6}.

Although interactions of plant lectins with a variety of animal cells and with different microorganisms are well documented (for reviews see refs 7 and 8), no information on their effect on fungi is available. We have found that WGA inhibits growth and spore germination of *T. viride*. These findings suggest a role for the lectin in nature, and provide further insight into the mechanism of fungal cell wall growth.

The binding of WGA to *T. viride* Pers. ex Fries isolate No. M 2042, was studied using fluorescein isothiocyanate-conjugated WGA (FITC-WGA). The lectin binds exclu-

sively to hyphal tips and septa, whereas most of the hyphae are not labelled (Fig. 1). The chitin in the septa and tips seems to be accessible to WGA whereas in the mature regions of the hyphae, the overlayering of chitin by glucans may prevent WGA binding. Effective labelling could be observed even after only a 10 s exposure of *T. viride* to a solution of 15 µg per 0.1 ml FITC-WGA. No labelling was observed when the colonies were preincubated with non-fluorescent WGA or when treated with FITC-WGA preincubated (30 min at 37 °C) with chitotriose (2 mM), a specific inhibitor of the lectin⁵.

³H-acetate has been used as a morphogenetic marker of intact *T. viride* colonies⁴. Short pulse labelling of colonies in the course of differentiation clearly labels the growing zones of the hyphae. Although acetate is not incorporated specifically into chitin, it is noteworthy that in young hyphae of *T. viride* chitinase removed all label from the cell wall after short ³H-acetate incorporation, thus demonstrating that most of the cell wall synthesis during short pulses is confined to production of chitin. As we had observed that WGA binds to hyphal tips, we studied the effect of the lectin on hyphal growth as indicated by ³H-acetate incorporation. Figure 2 demonstrates that as little as 125 µg WGA per 0.1 ml growth solution markedly inhibits ³H-acetate incorporation into hyphal tips, which is an indication of arrest of growth⁴. The incorporation of ³H-acetate was not affected in experiments in which WGA, preincubated with chitotriose (2 mM), was added to the colonies.

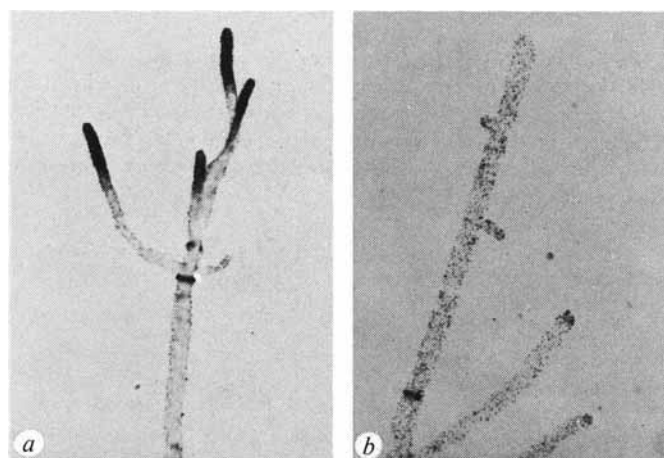
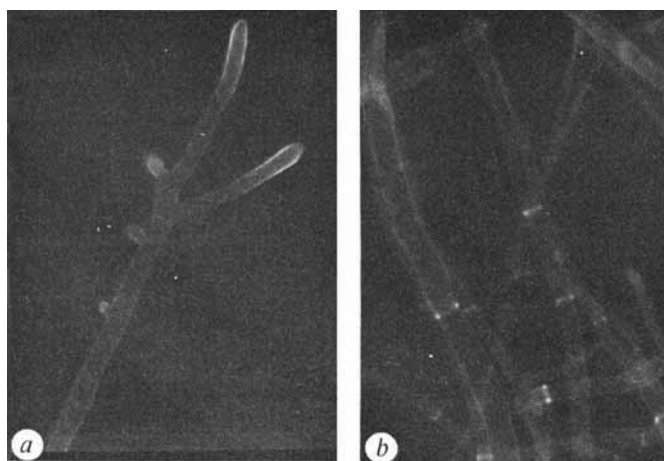


Fig. 2 Microautoradiographs of *T. viride* hyphae. Colonies of *T. viride* grown on slides⁴ were overlaid with WGA (30-500 µg in 0.1 ml minimal medium consisting of 10 g glucose, 0.5 DL-alanine, 1 g KH₂PO₄ and 0.5 g MgSO₄ in 1 l water). After incubation of 10 min at 25 °C, ³H-sodium acetate (18 Ci mmol⁻¹; 4 µCi in 0.1 ml minimal medium) was added on top of the WGA solution, and incorporation of label was allowed to proceed for 10 min. The slides were washed, fixed and processed for autoradiography as described⁴. *a*, Hyphae from a colonial front after incubation for 10 min with ³H-sodium acetate, normal optics. Note heavy labelling of hyphal tips and septum. *b*, Hyphae from a colony which was preincubated with 250 µg WGA and then labelled and photographed as in *a*. Note decrease in labelling at hyphal tip. Bars represent 20 µm.

Fig. 1 Microscopic appearance of *T. viride* hyphae treated with WGA labelled with fluorescein isothiocyanate. WGA was purified by affinity chromatography⁹ and conjugated with fluorescein isothiocyanate¹⁰. *T. viride* was cultured on 25 × 75 mm glass slides with frosted ends⁴. After 30-40 h growth in dark, FITC-WGA (50 µg in 0.1 ml phosphate buffered saline, pH 7.4) was spread over half of the colony and allowed to interact for 10 min at room temperature. Slides were then washed with saline and observed with a standard RA Zeiss fluorescence microscope. Exciter filter BG-12 was used in the path of the excitation light and barrier filter No. 53 in the path of the emitted light. *a*, Binding to hyphal tips; *b*, binding to septa. Bars represent 10 µm.



To obtain a more quantitative evaluation of the inhibition of ³H-acetate incorporation by WGA, we studied the effect with *T. viride* cultured by shaking in suspension. WGA markedly inhibited ³H-acetate incorporation into a suspension of young hyphae (60% inhibition at 0.8 mg ml⁻¹ WGA), whereas the incorporation of ³H-leucine was practically unaffected (Fig. 3a). In these experiments chitotriose (2 mM) also inhibited the effect of WGA.

To observe this possible inhibition of hyphal growth macroscopically, Petri dishes (9 cm diameter), containing potato dextrose agar were inoculated with *T. viride* and

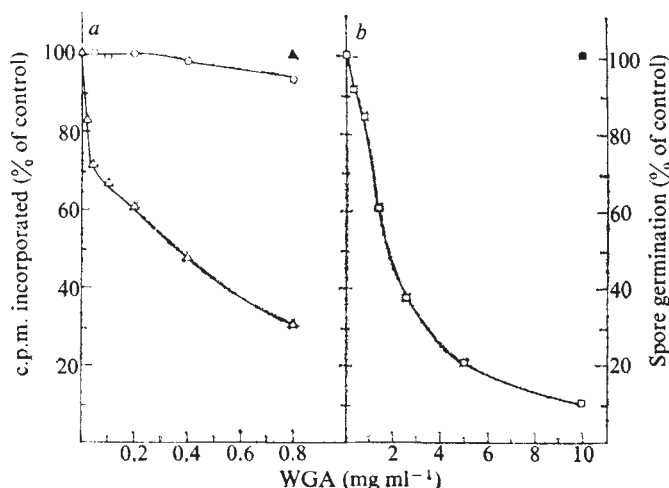


Fig. 3 *a*, Inhibition by WGA of ³H-sodium acetate incorporation into young hyphae. A suspension of young hyphae was obtained by allowing *T. viride* spores to germinate by shaking for 18 h in minimal medium at 25 °C. Aliquots (2 ml) of this suspension (0.27 mg dry weight ml⁻¹) were added to WGA solution (50–2,000 µg in 0.5 ml minimal medium) in 10 ml Ehrlemeyer flasks. After 15 min of shaking at 25 °C, ³H-sodium acetate (Δ18, Ci mmol⁻¹, 4 µCi in 0.1 ml minimal medium) was added to each flask and the flasks were shaken for a further 5 min. The pulse labelling was terminated by addition of 3 ml cold solution CCl₃COOH (10% w/v) and the flasks kept in the cold for 15 min. Suspension was filtered over GF/C glass filters (Whatman) and washed with 20 ml 1 N acetic acid. Filters were dried for 2 h at 100 °C and the radioactivity remaining counted in toluene scintillation liquid with an efficiency of 52%, using a Packard Tri-Carb scintillation spectrometer. In parallel experiments, the incorporation of ³H-leucine (19 Ci mmol⁻¹, 4 µCi in 0.1 ml) was followed in the same conditions. Percentage inhibition was calculated from the ratio of the radioactivity incorporated in the presence of WGA to that incorporated in its absence. ▲, Obtained when WGA was preincubated (30 min at 37 °C) with chitotriose (2 mM). *b*, Inhibition by WGA of *T. viride* spore germination. WGA (10–300 µg in 15 µl minimal medium) was mixed with *T. viride* spores (15 µl suspension of 0.27 mg dry weight per ml in minimal medium) and the spores were allowed to germinate for 18 h at 25 °C. Aliquots (25 µl) were spread on a microscope slide and germination was observed under the microscope. Percentage germination was calculated after counting 1,000 spores germinated in the presence and absence of WGA. ■, Germination in presence of WGA and 2 mM chitotriose.

kept in the dark at 25 °C. When the colonies reached a diameter of 4 cm (after 2 d), they were exposed to laboratory light; three wells were drilled in the agar, 1 cm from the front of the colony (3 cm from the centre of the plate) and filled with WGA (30–500 µg in 50 µl minimal medium) and the colonies were allowed to grow (4 d) until they covered the dish. Exposure to light induced conidiation¹¹ and green rings of conidia marked the pattern of the colonies' extension. As little as 60 µg WGA in a well inhibited hyphal extension (Fig. 4*a* and *b*). The area of the clear zone around the wells, where growth was inhibited, was concentration-dependent. The specificity of the effect is indicated by the normal growth of the fungus when WGA was placed in wells in the presence of the WGA-inhibitors, chitotriose (2 mM) or teichoic acid of *Staphylococcus aureus* H (0.6 mM), (Fig. 4*c*). The latter is a very potent inhibitor of WGA (ref. 12).

It has been shown that the cell wall of hyphal tubes emerging out of germinating spores of the fungal group V (ref. 1), to which *T. viride* belongs, contain chitin³. Indeed, a marked inhibition of spore germination was observed when WGA was mixed with spores of *T. viride* (Fig. 3*b*). The effect of WGA in inhibiting the germination of the spores of these fungi seems to be the result of its binding to the chitin at the tip of the emerging hyphal tube rather than to the spore coat, as no binding of FITC-WGA to the spores could be observed. Furthermore, the spores were not agglutinated by WGA.

The evidence presented above indicates that binding of WGA to hyphal tips inhibits chitin synthesis, hyphal growth and spore germination. Although the mechanism of apical growth is poorly understood, it has been suggested that the addition of newly synthesised chitin for extension of the hyphal cell wall and the onset of germination in spores, require the cleavage of pre-existing chitin in the hyphal tip by selective lysis³. Binding of the multivalent WGA (refs 13 and 14), to the chitin at the hyphal tip may result in cross-linking of the latter and thus inhibit the extension process. The perturbation of the delicate balance between wall synthesis and wall lysis which presumably controls apical wall growth³ may cause the arrest of chitin synthesis. The effect of WGA seems to be of a fungistatic nature as it does not cause inhibition of leucine incorporation during short pulses (Fig. 3*a*).

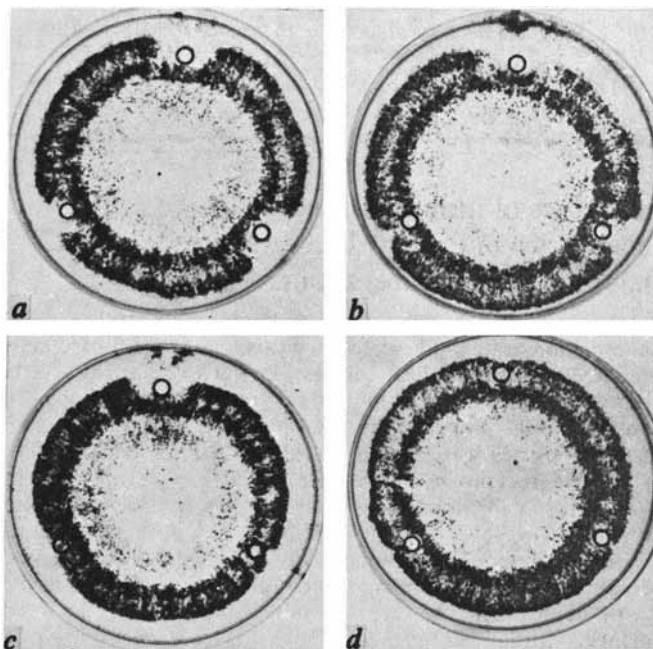


Fig. 4 Inhibition of conidiation and arrest of *T. viride* hyphal extension by wheat germ agglutinin. *T. viride* was grown on potato dextrose agar in Petri dishes. When the colonies reached a diameter of 4 cm, WGA was placed in wells drilled in the agar 1 cm from the front of the colony and the colonies were allowed to grow further. The wells on top, right, and left contained: *a*, 500 µg, 250 µg and 125 µg WGA, in 50 µl minimal medium, respectively; *b*, 125 µg, 60 µg and 30 µg WGA respectively; *c*, top well contained 250 µg WGA, right-hand well contained the same amount of lectin together with 1 mg purified teichoic acid of *S. aureus* H, left-hand well contained 250 µg WGA and 2 mg chitotriose; *d*, all the wells contained minimal medium (50 µl).

A possible role of plant lectins in attaching nitrogen-fixing bacteria to plant roots has been proposed^{15,16}. In view of the experiments described in this paper, as well as our finding that FITC-WGA binds to the hyphal tips of *Fusarium solani* (Mart.) Sacc. and inhibits ³H-acetate incorporation into their cell wall, it is tempting to suggest a role for this lectin in nature. It is possible that WGA protects wheat against chitin-containing phytopathogens during seed imbibition, germination and early seedling growth. The role of lectins in protecting plant seeds in which their concentration is usually high, may be a general one. Accordingly, lectins with sugar specificities which differ from that of WGA may inhibit the growth of other soil microorganisms, the surface of which are covered by polysaccharides such as glucans, galactans, mannans, or various heteropolysaccharides¹.

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Inhibition of mobility of cell-surface receptors by factors which mediate specific cell-cell interactions

PREVIOUS studies on embryonic chick neural retina cells¹ have demonstrated that cells in intact tissues or single cells prepared by mechanical dispersion have relatively little ability to redistribute their surface receptors into caps. In contrast, cells prepared by trypsinisation and dissociation of intact tissues were found to exhibit lateral redistribution of surface receptors even in the absence of a multivalent ligand. After 4 h culture, however, cells dissociated by trypsinisation have lost the capability for both spontaneous and lectin-induced rearrangements of surface components. Mobility of cell-surface receptors thus seems to be related to, or determined by, trypsin-labile components at the cell surface. One kind of component which seems to be trypsin labile is also important in intercellular recognition and adhesion^{2,3}. We now report that the binding of tissue-type specific cell-surface ligands which seem to mediate recognition and adhesion causes inhibition of directed rearrangement of plant lectin receptors in the plane of the membrane.

Procedures for collection of aggregation-promoting supernatant solutions (APMs), retina aggregation promoting material (RAPM) and cerebral lobe aggregation promoting material (CLAPM) were as described previously⁴. The assay for redistribution of fluorescent-labelled lectin receptors on the surface of 10-d embryonic chick neural retina cells freshly dissociated from tissues by trypsinisation was carried out as described previously¹. The same procedure was used for 10-d cerebral lobe cells. Fluorescein-isothiocyanate conjugated concanavalin A (FITC-con A), wheat germ agglutinin (FITC-WGA) and soybean agglutinin (FITC-SBA) were purchased from Miles. Cells labelled with fluorescent lectin were examined under a Zeiss Universal microscope using an epifluorescence system and $\times 40$ oil-immersion objective. Protein determinations on dialysed aliquots of RAPM and CLAPM were carried out by the method of Lowry *et al.*⁵.

As shown in Fig. 1, RAPM inhibits cap formation of FITC-con A receptors on trypsin-dissociated 10-d chick neural retina cells. The effect depends on concentration with a plateau value at approximately 80% inhibition at RAPM concentrations greater than 25 $\mu\text{g ml}^{-1}$ protein. Added CLAPM or foetal calf serum (Gibco) have a

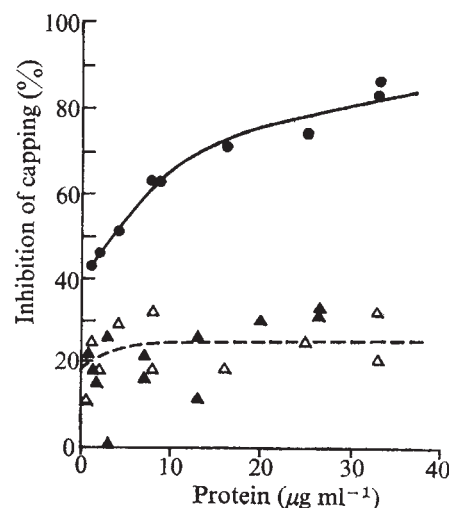


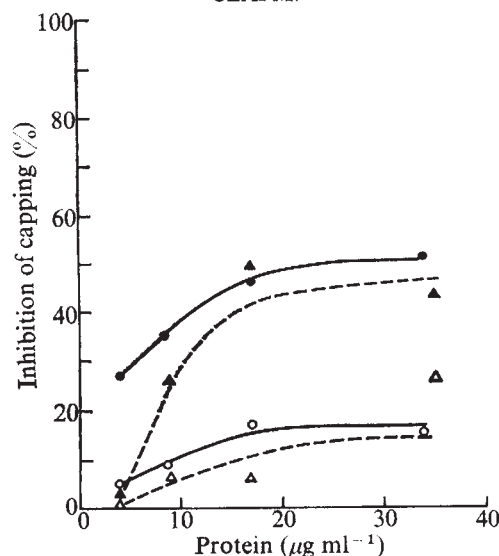
Fig. 1 Effect of APMs and foetal calf serum on cap formation of con A receptors in 10-d chick neural retina cells. Cells (20×10^6) prepared by trypsinisation were incubated with 60 μg FITC-con A in 1 ml Eagle's basal medium for 20 min at 0°C , followed by incubation for 60 min at 22°C with or without additives, pelleted at 200g for 5 min, and fixed in 2% glutaraldehyde (pH 7.4) for 30 min at 0°C . ●, RAPM; ○, CLAPM; ▲, foetal calf serum.

maximal effect of approximately 25% inhibition of capping over the same protein concentration range. None of these preparations had any effect on patch formation.

The inhibitory effect of RAPM on surface receptor mobility is not limited to the con A receptors. Figure 2 illustrates that receptors for both FITC-WGA and FITC-SBA are also rendered increasingly immobile after treatment with various concentrations of RAPM, although the maximal inhibition of capping by RAPM only reaches a level of approximately 50% for these receptors. As is the case for the con A receptors, CLAPM causes much less inhibition of capping of WGA and SBA receptors, with maximal inhibition of 15-20%.

To examine the tissue-type specificity of the effect of APMs on lectin receptor mobility, 10-d chick cerebral lobe cells were used in otherwise identical experiments. In this case, CLAPM inhibits cap formation of con A receptors in a concentration-dependent manner to a level of approximately 80%. The maximal inhibition obtained with

Fig. 2 Inhibition by APMs of cap formation of wheat germ agglutinin (●, ○, solid lines) and soybean agglutinin (▲, △, dashed lines) receptors in 10-d chick neural retina cells. Experimental conditions as described in Fig. 1. ●, ▲, RAPM; ○, △, CLAPM.



RAPM on cerebral lobe cells is 25% (Fig. 3). These results suggest that RAPM and CLAPM are able to specifically immobilise receptors on the cell type from which they originate.

Control of the mobility of one surface component by another has also been reported in lymphocytes^{6,7}. In this system, incubation of cells with concentrations of con A greater than $5 \mu\text{g ml}^{-1}$ causes inhibition of cap formation of surface immunoglobulin molecules. Drugs which are thought to disrupt microtubules, however, were able to reverse the inhibition by con A. To examine whether inhibition of the mobility of lectin receptors by RAPM is an analogous situation, cytochalasin B (Aldrich, $20 \mu\text{g ml}^{-1}$ in dimethyl sulphoxide) or colchicine ($40 \mu\text{g ml}^{-1}$, Sigma) were added to retina cells being assayed for receptor redistribution with FITC-con A. No reversal of the inhibition by RAPM was observed. Moreover, we have previously observed¹ that treatment with these drugs does not overcome to the inhibition of con A receptor mobility in the presence of excess con A.

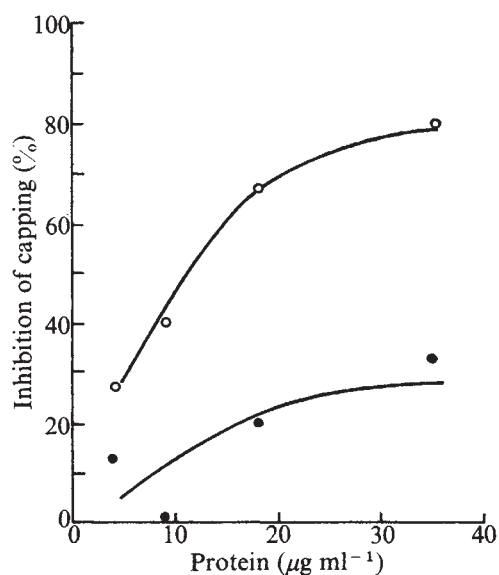


Fig. 3 Inhibition by APMs of cap formation of con A receptors in 10-d chick cerebral lobe cells. Experimental conditions as described in Fig. 1. ●, RAPM; ○, CLAPM.

It has previously been demonstrated in this laboratory that binding of RAPM to retina cells depends on a terminal N-acetyl-galactosamine residue, whereas binding of CLAPM to cerebral lobe cells depends on a terminal mannosamine residue⁸. To establish a positive correlation between binding activity and inhibition of surface component redistribution activity, RAPM was treated with purified N-acetyl-hexosaminidase (Miles) and CLAPM with purified α -mannosidase (Miles). Enzyme-treated RAPM preparations have a decreased ability to inhibit con A receptor mobility on neural retina cells that almost exactly corresponds to the loss of binding activity (Table 1). Similarly, CLAPM treated with α -mannosidase shows a parallel decrease in ability both to bind to cells and to inhibit con A receptor mobility of cerebral lobe cells (Table 1). Thus, by the criteria of both specificity of action and behaviour after enzyme treatment, it seems that the portion of the APM preparation which has binding activity is very similar to, or identical with, the portion which inhibits surface receptor mobility.

Taken together, these results suggest that supernatant preparations from cultures of 10-d chick neural retinae or cerebral lobes enhance reaggregation of dissociated single cells in a tissue-type specific fashion^{3,9}, specifically bind to such cells⁴, and are also able to specifically induce an alteration in some membrane-related parameter, the effect of

Table 1 Comparison of loss of binding activity with loss of inhibition of lectin receptor redistribution activity after glycosidase treatment

APM	Preparation	Loss of binding activity (%)	Loss of inhibition of lectin receptor redistribution activity (%)
RAPM	1	20	14
	2	40	35
	3	60	70
CLAPM	1	10	26
	2	50	45
	3	70	63

RAPM was incubated in 0.05 M sodium acetate buffer (pH 4.0) at 37°C for 30 min with 0.10, 0.25, and 0.45 U ml^{-1} purified N-acetyl-hexosaminidase (Miles) for preparations 1, 2, and 3, respectively. Enzyme-treated RAPM was boiled for 10 min and dialysed overnight against Tyrode's solution. CLAPM was treated with purified α -mannosidase at concentrations of 0.01, 0.03, and 0.04 U ml^{-1} for preparations 1, 2, and 3, respectively, using the conditions above with the exception that the incubation was for 60 min. Boiled enzyme controls were done in each experiment. Binding to cells was carried out as described previously⁴. Experimental conditions for the assay of inhibition of lectin receptor mobility were as described in the legend to Fig. 1. RAPM was assayed on 10-d chick neural retina cells, CLAPM on 10-d chick cerebral lobe cells.

which is to inhibit directed rearrangements of surface receptors into caps. As receptors for three different lectins which have been shown to redistribute independently in the plane of the membrane¹ are all affected in basically the same manner by APMs, we infer that the inhibition is a general one, affecting most or all of the cell-surface components.

Control of cell-surface topography by ligands specific for tissue type may be of fundamental importance to the processes of cell recognition, adhesion and histotypic rearrangement. Whether or not neighbouring cells form stable contacts may depend, at least partially, on the distribution of surface components important for recognition and adhesion. The action of such ligands at the surface of each cell may be to maintain the surface of appropriate neighbouring cells in a configuration most suitable for stable contacts. A similar hypothesis has been advanced by Bennett *et al.*¹⁰ which suggests that epigenetic control of cell-surface architecture is an important element in determination and morphogenesis. The combined use of general surface markers, such as lectin probes, with tissue-type-specific surface markers, such as APMs, should enable exploration of this hypothesis in normal morphogenetic contexts.

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Spleen or thymus cell suspensions of BALB/c mice in a total volume of 0.15 ml (2×10^6 cells ml⁻¹) were cultured in Microtitre plates M24AR, system Cooke (C. A. Greiner, Nürtingen, Germany) in an atmosphere of 5% CO₂ in air at 37 °C for 48 h in RPMI1640 medium supplemented with 5% fresh heat-inactivated human serum, 2 mM L-glutamine, 100 U penicillin and 100 µg streptomycin ml⁻¹ (culture medium). ³H-thymidine (0.1 µCi; 2 Ci mM⁻¹; Radiochemical Centre, Amersham) was added to the cultures for the last 4 h of incubation period. Cells were collected on glass fibre filters using a Skatron multiple cell culture collector. Incorporation of ³H-thymidine into the nuclear DNA was then determined as described previously⁴. Non-adherent spleen cells, containing less than 1% macrophages, were obtained using a glass bead column as described previously¹³. Peritoneal cells (PC) of BALB/c mice were obtained by injecting 5 ml culture medium intraperitoneally and aspirating the fluid from the peritoneal cavity 5 min later. The cell population obtained consisted of ~75% monocytes, 10% polymorphs, and 15% lymphocytes. Cyclic nucleotides were obtained from Boehringer, Mannheim; LPS (lipopolysaccharide of *E. coli* 055:B5) and PHA from Difco, Detroit. Results represent arithmetic means of c.p.m. detected in triplicate cultures, s.e. less than 10%. As determined by separate experiments the highest number of PC (3×10^4 cells) added to the cultures gave a negligible response to mitogens (less than 30 c.p.m.)

Table 2 3',5'-cyclic GMP-dependent release of lymphocyte-activating factor from peritoneal cells of mice

Source of supernatant	Stimulation index	
	Thymocytes	Spleen cells
PC + 3',5'-cyclic GMP	12.0 (23.2)	3.0
PC + 2',3'-cyclic GMP	0.9	1.1
PC + 3',5'-cyclic AMP	0.7	1.0
PC + 3',5'-cyclic GMP	11.0	2.8
+ 3',5'-cyclic AMP		
PC + 3',5'-cyclic GMP	13.0	2.8
+ EGTA		

Peritoneal cells (PC) of BALB/c mice were incubated in a culture medium (see Table 1) with either 3',5'-cyclic GMP (5×10^{-3} M), 2',3'-cyclic GMP (1×10^{-3} M), 3',5'-cyclic AMP (1×10^{-3} M), or a combination of 3',5'-cyclic GMP (5×10^{-3} M) and 3',5'-cyclic AMP (1×10^{-3} M), or a combination of 3',5'-cyclic GMP (5×10^{-3} M) and EGTA (0.6 mM) (see Table 1). Culture fluids were collected after 24 h and sterilised by filtration (Millipore filters). Thymocytes or spleen cells (2×10^6 ml $^{-1}$) of BALB/c mice were then cultured as described in Table 1 in the presence of 10% various supernatants (final concentration). Incorporation of 3 H-thymidine into the nuclear DNA was determined in 48 h cultures. Results are expressed as stimulation indices (c.p.m. in cultures in presence of 10% supernatant of PC cultures/c.p.m. in cells cultured without addition of supernatant). Each value represents mean of triplicate cultures, s.d. less than 10%. Value in brackets shows response of thymocytes to same supernatant after extensive dialysis against culture medium (without serum) for 24 h.

adherent cells. 3',5'-cyclic GMP induces the release of a soluble non-dialysable factor from peritoneal cells with lymphocyte (predominantly thymocyte)-activating properties. The factor seems to be similar or identical with the lymphocyte activating factor already described^{7,9}, indicating the possibility that the production of the factor is mediated by a 3',5'-cyclic GMP-dependent process.

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Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier

THE most exciting aspect of immunology at the moment is the way in which the major histocompatibility complex regulates certain functions of the immune system. Understanding the basis of this will revolutionise our thinking about T cell responsiveness and perhaps explain the puzzling degree of genetic polymorphism of the major histocompatibility complex. Most rewarding also will be the clinical applications, since susceptibility to many diseases is linked to HL-A (refs 1 and 2). Genes which control the response of thymus-derived (T) lymphocytes to certain antigens map in the major histocompatibility complex¹; 1–10% of T lymphocytes respond in

an allogeneic mixed lymphocyte culture, or graft-versus-host reaction, against non-self major histocompatibility antigens^{3–5}; cytotoxic T cells derived from mixed lymphocyte cultures seem to be directed against the classical serologically defined antigens coded in this region^{6,7}. The work described here derives from another recently reported link between murine T cell function and the major histocompatibility complex. It has now been demonstrated that T-cell mediated lysis of virus infected target cells is restricted by H-2 (refs 8–10). Lysis of TNP modified

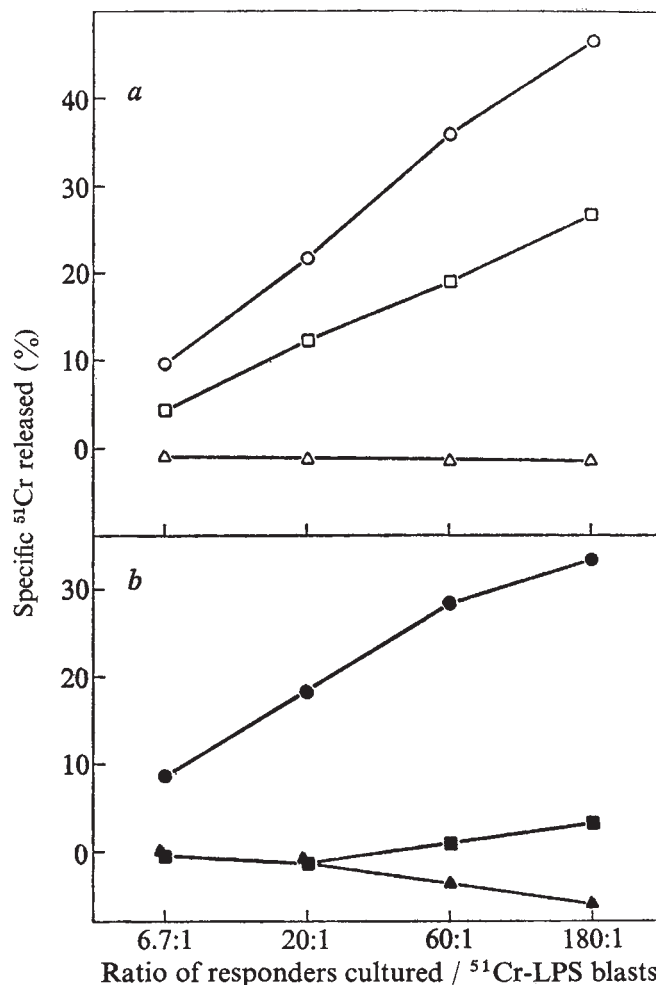


Fig. 1 Assay of lysis of six targets by BALB/c(H-2^d) anti-B10.D2(H-2^d) cytotoxic T cells. Normal BALB/c mice were immunised by intraperitoneal injection of 44×10^6 spleen and lymph node cells from B10.D2 mice in Hanks' balanced salt solution. The spleen was removed 18 d later and 4×10^6 viable cells ml $^{-1}$ were restimulated *in vitro* with 4×10^6 cells ml $^{-1}$ irradiated (1,000 rad from a ^{60}Co source) B10.D2 spleen cells. Responder and stimulator cells were from female mice. After 4 d the cultures were collected and washed once before the assay. Targets were spleen cells which had been cultured for 3 d in the presence of $10 \mu\text{g ml}^{-1}$ LPS to induce blast transformation of the B cells^{7,13}. Targets were collected, washed, labelled with ^{51}Cr -sodium chromate, washed twice, and viable blasts counted. Threefold dilutions of the responder cells were titred against a constant number (5×10^4) of ^{51}Cr -labelled LPS blasts for 4 h in 35-mm Petri dishes on a rocking platform. At the end of the assay, the contents of the dishes were transferred to tubes, centrifuged, and half the supernatant removed for counting. The results are plotted as the ratio of responders originally cultured to 5×10^4 ^{51}Cr -targets (logarithmic scale) against the percentage specific ^{51}Cr -release (arithmetic scale) calculated as:

$$\frac{\text{Experimental release} - \text{spontaneous release}}{100 - \text{spontaneous release}} \times 100$$

Targets were: B10.D2(H-2^d) (○); DBA/2(H-2^d) (□); B10.BR(H-2^k) (△); C57BL/6(H-2^b) (●); B6.C/H-2^d (synonym HW19) (◐); and BALB/c(H-2^d) (▲). Spontaneous release (release by 5×10^4 targets cultured alone) varied from 18.2 to 25.9%.

targets is similarly restricted in another system¹¹. I have now shown that cytotoxic T cells derived from mice immunised with cells from an allogeneic strain which carries the same H-2 region will lyse only targets which share the same H-2 as the immunising strain.

Schematically the results can be summarised as follows: cytotoxic T cells of strain A(H-2¹) immunised against cells of strain B(H-2¹) will lyse B(H-2¹) targets but not B(H-2²) targets; they do, however, crossreact strongly and kill cells of strains C(H-2¹) or D(H-2¹) but not C(H-2²) or D(H-2⁴). Such cytotoxic target antigens apparently include hybrid specific antigens (that is, antigens present on an F₁ but not on either homozygous parent). Schematically, A(H-2¹) anti-B(H-2¹) will not lyse A(H-2¹) nor B(H-2²) but will lyse F₁(A(H-2¹) × B(H-2²)) targets. I favour the hypothesis that all the allogeneic surface structures which are targets for cytotoxic cells in this system are interaction antigens created by an H-2 coded modification of the products of non-H-2 coded genes—probably minor histocompatibility genes.

The immunisations were done by priming the mice with an intraperitoneal injection of living allogeneic H-2 similar stimulating cells and 18–40 d later removing the spleen and boosting *in vitro* with irradiated stimulating cells. All the cytotoxic effector activity generated has been shown to be sensitive to treatment with anti-Thy-1.2 serum and complement immediately before the assay. Figure 1 shows the lytic activity of BALB/c (H-2^d) anti-B10.D2 (H-2^d) cells against targets from six strains of mice. Cells syngeneic with the responders were not lysed (Fig. 1b). B10.D2 was lysed very efficiently, but B10.BR (H-2^k) which is genetically the same as B10.D2, except for the H-2 region, was not lysed (Fig. 1a). Targets from another unrelated strain, DBA/2, which carries H-2^d were killed quite efficiently (Fig. 1a). A trivial explanation of this cross reaction was that, since B10.D2 derived its H-2^d region from DBA/2, the BALB/c cells were immunised against a surface alloantigen closely linked genetically to, or within, the DBA/2 and B10.D2 H-2 region. This explanation is ruled out in Fig. 1b, which shows the results with another congenic pair of targets. C57BL/6 (H-2^b) was lysed only very weakly by this population of BALB/c anti-B10.D2 cytotoxic cells, whereas B6.C/H-2^d which is congenic with C57BL/6 and derived H-2^d from BALB/c¹², was lysed very efficiently, about as well as B10.D2 targets.

Figure 2 shows the activity of BALB/c and B10.D2 cytotoxic cells obtained after immunisation with P815, a DBA/2 (H-2^d) mastocytoma, when assayed at the same time against targets from six strains of mice. Neither population caused lysis of syngeneic targets. Both lysed cells of the immunising (DBA/2) strain very efficiently (Fig. 2a and b). BALB/c anti-P815 cross-reacted strongly on B10.D2, as would be predicted from Fig. 1,

but did not lyse B10.BR(H-2^k) (Fig. 2a); B10.D2 anti-P815 on the other hand crossreacted strongly on BALB/c targets (Fig. 2b). The BALB/c cytotoxic population did not lyse syngeneic targets, and only caused slight lysis of C57BL/6 (H-2^b), but F₁ (C57BL/6 × BALB/c) targets were lysed very well (Fig. 2a). Thus the cytotoxic T cells detected antigens found only on F₁ cells but not either parent, implying the existence of hybrid specific antigens.

In the experiment shown in Fig. 2a, BALB/c anti-DBA/2 cytotoxic cells lysed B10.D2 (H-2^d) targets efficiently but not B10.BR(H-2^k) targets, even though the two strains are congenic and differ only at H-2. It seemed possible that B10.BR did express antigens which the cytotoxic cells recognised on B10.D2, but the lytic function was somehow impaired by the different H-2 antigens. The question was thus whether the lysis of ⁵¹Cr-labelled B10.D2 targets caused by BALB/c anti-DBA/2 cytotoxic cells could be inhibited by the addition of unlabelled B10.BR cells. In this "cold target competition" experiment, damage of B10.BR cells was not measured, only their capacity to be bound by receptors on the cytotoxic cell^{7,14}. As a control, unlabelled targets of BALB/c (syngeneic with the cytotoxic cells) or B10.D2 (syngeneic with the labelled target) were used as inhibitors. Table 1 shows that an 8 or 33-fold excess of B10.BR cells over ⁵¹Cr-B10.D2 cells did not inhibit release of label any more than did the same excess of BALB/c cells. Unlabelled B10.D2 naturally inhibited lysis. This is strong evidence that the cytotoxic cells did not see the same non-H-2 coded antigens on B10.BR as they did on B10.D2.

Two hypotheses will now be considered to explain this finding that, even after an allogeneic immunisation when responder and stimulator share the same H-2, there is a requirement in lysis for the target also to share the same H-2. The first is that there is dual recognition by the cytotoxic effector cell of H-2 coded and non-H-2 coded structures on the target cell surface. This could mean that the cytotoxic cell receptor for non-H-2 is clonally restricted but all cells bear receptors for H-2, that is, a requirement for self-H-2 recognition. There are four lines of evidence against this. First, Zinkernagel and Doherty showed that F₁ cells immune to LCM-infected F₁ cells, after boosting in parent 1 virus-infected hosts, killed infected cells of parent 1 but not of parent 2 (ref. 15). Second, I have performed a similar experiment in which F₁ (BALB/c × BALB.B) (H-2^{d/b}) immune to B10.D2 (H-2^d) did lyse B10.D2, but did not lyse B10 (H-2^b). Third, the cold target competition experiment reported here (Table 1) shows that B10.BR cells fail to inhibit the binding of BALB/c anti-DBA/2 cytotoxic T cells to B10.D2 targets. Fourth, cytotoxic cells with specificity for foreign H-2 exist, with no apparent requirement in lysis for recognition of other surface structures. Alternatively, both the cytotoxic cell re-

Table 1 Inhibition by unlabelled cells of the lysis of ⁵¹Cr-B10.D2 targets mediated by BALB/c anti-DBA/2 cytotoxic cells

Unlabelled LPS blasts used for inhibition*	H-2	Genotype: background	Experimental-spontaneous release†	Factor of inhibition of lysis‡
None	—	—	37.7	—
BALB/c (×8)	d	BALB/c	32.4	1.0
BALB/c (×33)	d	BALB/c	29.3	1.0
B10.BR (×8)	k	B10	32.3	1.0
B10.BR (×33)	k	B10	30.9	0.9
B10.D2 (×8)	d	B10	17.7	3.2
B10.D2 (×33)	d	B10	6.3	13.6

BALB/c mice were immunised by intraperitoneal injection of 10⁷ P815 and by a secondary boost *in vitro* with 5,000 rad irradiated P815. These cytotoxic BALB/c anti-DBA/2 cells were titred against 5 × 10⁴ ⁵¹Cr-labelled B10.D2 LPS blasts for 4 h at ratios of from 43:1 to 0.18:1. At a ratio of 12:1, an 8-fold (4 × 10⁶), or 33-fold (1.65 × 10⁶) excess of unlabelled LPS blasts from BALB/c, B10.BR or B10.D2 were added to the assay to inhibit ⁵¹Cr release.

*Numbers in parentheses refer to the numerical excess of unlabelled LPS blasts over ⁵¹Cr-B10.D2 blasts present in the assay.

†Spontaneous release was 34.8%.

‡A factor of 1.0 refers to no inhibition relative to the same excess of BALB/c LPS blasts. An 8-fold excess of BALB/c blasts in fact gave a 1.8-fold inhibition, and a 33-fold excess a 2.4-fold inhibition compared with when no unlabelled inhibitor cells were present. The factor of inhibition was calculated from the titration curve as described previously⁷.

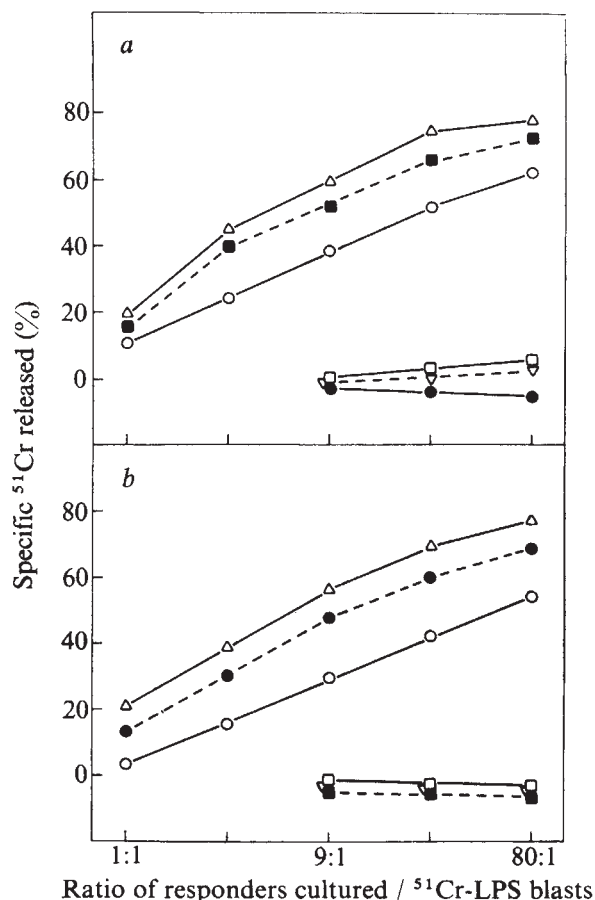


Fig. 2 Lysis of six targets by cytotoxic T cells from: *a*, BALB/c (H-2^d) and *b*, B10.D2(H-2^d) mice immunised against DBA/2 (H-2^d) cells. Responder mice were injected intraperitoneally with 10⁷ viable DBA/2 mastocytoma cells, P815, which were grown in tissue culture. 20 days later the spleen was removed and cultured at 4 × 10⁶ cells ml⁻¹ with 2 × 10⁵ cells ml⁻¹ irradiated (5,000 rad from a ⁶⁰Co source) P815. After 4 d the cells were washed and threefold dilutions titred against ⁵¹Cr-labelled LPS blast targets as in Fig. 1. Targets were: DBA/2(H-2^d) (Δ); B10.D2(H-2^d) (■); C57BL/6(H-2^b) (□); B10.BR(H-2^k) (▽); BALB/c(H-2^d) (●); and F₁(C57BL/6 × BALB/c) (○). Spontaneous release varied from 24.2 to 31.4%.

ceptor for non-H-2 and for H-2 may be clonally restricted. Evidence against this includes the third and fourth arguments above.

Hypothesis 1 is rejected at this stage of experimentation in favour of the second. This states that the H-2 region codes for a product which interacts with the products of genes coded elsewhere, and the result of this is the creation of new complex determinants (interaction antigens) which are recognised by allogeneic cytotoxic T cells. Although viral infection may alter H-2 antigens¹⁶, the targets in my work are normal cells and a wealth of previous literature^{6,17} and one preliminary experiment performed in this laboratory do not suggest a difference in the H-2 antigens on BALB/c against those on DBA/2 or B10.D2 which are recognised by anti-H-2^d cytotoxic cells. I therefore favour the explanation that H-2 codes for a modifier of all the other surface components which are targets in this system. In a hybrid, such as F₁ (C57BL/6 (H-2^b) × BALB/c (H-2^d)), the maternal (C57BL/6) and paternal (BALB/c) non-H-2 coded components are modified by both the maternal (H-2^b) and paternal (H-2^d) H-2 complex. This results in the creation of hybrid specific alloantigens, that is, the H-2^d (paternal) modification of the C57BL/6 (maternal) non-H-2 coded components and the H-2^b (maternal) modification of the BALB/c (paternal) non-H-2 coded components (Fig. 2a). The strong cross reaction shown here between unrelated strains with the same H-2 haplotype can be explained if the non-H-2 components are coded by minor

histocompatibility genes. Strains differ by a large number of minor histocompatibility loci but the number of alleles at each locus is small¹⁸. The basis for the cross reaction between DBA/2 and BALB/c detected by B10.D2 (Fig. 2b) is explicable if the two strains share the same allele at some minor histocompatibility loci where B10.D2 carries a different allele.

In terms of the molecular nature of the H-2 coded modification, the most attractive idea at the moment is that the major histocompatibility complex codes for a glycosyl transferase system which uses as substrate the products of genes which code for surface components (P. Trefts and B. E. Rothenberg, unpublished). This predicts that in allogeneic T-cell mediated killing the target antigens are carbohydrate in nature.

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Interchange of adenyl and guanyl cyclases as an explanation for transformation of β- to α-adrenergic responses in the rat atrium

STUDIES have indicated that the response of the heart¹⁻⁵, and possibly other tissues⁶, to exogenous catecholamines changes from β-type to α-type in conditions of decreased metabolic activity, including lowering the temperature. Attempts to correlate this with changes in cyclic AMP metabolism which are thought to reflect β-adrenoceptor activity were unsuccessful^{7,8}. We have therefore studied the effects of lowering the temperature on the intracellular levels of both cyclic AMP and cyclic GMP in the spontaneously beating rat atrium. Cyclic GMP concentrations seem to mediate α-adrenergic responses in a variety of systems⁹⁻¹², and we therefore investigated whether its levels increased in conditions of increased α-adrenoceptor activity. Our results indicate that the same receptors that stimulate cyclic AMP synthesis at 37 °C, trigger the synthesis of cyclic GMP at a colder temperature (24 °C).

Figure 1 shows that the changes in cyclic nucleotide concentration induced by the low optimal concentrations of three catecholamines preceded their chrono- and inotropic effects. Increases in cyclic GMP levels were more prominent at 24 °C than at 37 °C, whereas changes in cyclic AMP were not impressive and confirm the previously observed lack of correlation between cyclic AMP levels and β-adrenoceptor activity at low agonist concentrations¹³.

When peak changes in rate × force were used as an index of atrial performance, lowering the temperature to 24 °C resulted in transformation of β to α-adrenoceptors (Fig. 2).

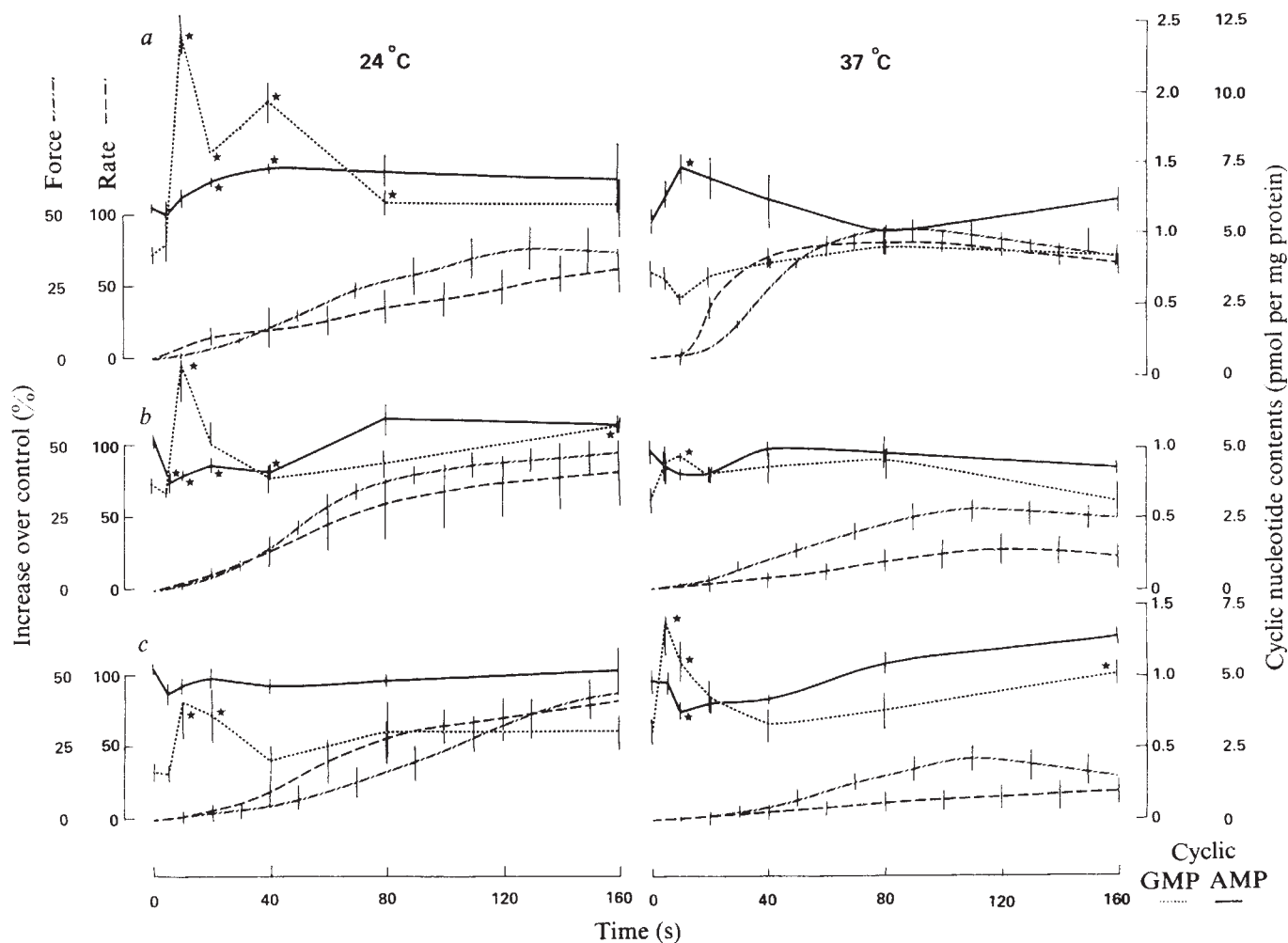


Fig. 1 Time course of effect of three catecholamines on rat atrial performance and cyclic nucleotide contents at 24 and 37 °C. *a*, Isoproterenol; *b*, adrenaline; *c*, noradrenaline. Right and left atria were removed together from 150–200 g male Sprague-Dawley rats and suspended in a tissue bath containing 25 ml Chenoweth's solution¹⁴ aerated with 5% CO₂ in O₂. Bath temperature was maintained at 24 or 37 °C throughout. Right atrial appendage was fixed in the bath, left atrial appendage was attached by a fine silk thread to a Grass FT03 force transducer, and contractile activity was recorded with a Grass model 7 polygraph. Diastolic tension was maintained at 2 g. Atria were allowed to equilibrate for 30 min. Spontaneous heart rate and contractile force were obtained from the record and were 98–125 beats min⁻¹ and 0.82 to 0.97 g at 24 °C and 286–304 beats min⁻¹ and 0.28 to 0.37 g at 37 °C, respectively, immediately before the additions of the agonists (10⁻⁷ M) which were added to the bath at zero time. To determine cyclic nucleotide levels, atria were quickly frozen at the predetermined times using modified Wollenberger clamps previously cooled in liquid nitrogen. Atria were kept frozen at -70 °C until analysed for cyclic nucleotide contents not more than 2 weeks later. The two cyclic nucleotides were separated from each other on Dowex-formate columns¹⁵. Cyclic AMP was determined by the protein binding method of Gilman¹⁶ and cyclic GMP was determined by radioimmunoassay¹⁷. Internal standards of tritiated cyclic nucleotides were used to calculate recoveries, which were 87–95% for cyclic AMP and 55–78% for cyclic GMP. Phosphodiesterase treatment destroyed all assayable cyclic nucleotides. Data for both atrial performance and cyclic nucleotide contents are the average of six experiments $\pm$ s.e.m. — —, Percentage change in rate; — · — · —, percentage change in force; — — —, cyclic AMP contents; · · · · ·, cyclic GMP contents. *Statistical significance from corresponding zero time control.

This is shown by the disappearance of the order of potency, isoproterenol > adrenaline or noradrenaline, characteristic of the β -receptor; and the greater susceptibility of the catecholamine effects to blockade by the α -adrenoceptor blocker phentolamine, which is ineffective at 37 °C; at this temperature propranolol or sotalol (not shown) were the more effective blockers. Similar results were obtained when rate alone instead of rate $\times$ force was used as an index of atrial function, except that the effects of noradrenaline in the cold were less susceptible to blockade by phentolamine. These data agree well with those reported for frog and rat hearts^{1–4}.

At 37 °C cyclic AMP levels seemed to reflect β -adrenoceptor activity, as isoproterenol was more effective than adrenaline and noradrenaline in raising this cyclic nucleotide level, particularly at the higher agonist concentration (Table 1). In addition the changes in cyclic AMP induced by isoproterenol and adrenaline were blocked by the β -adrenergic blocker propranolol, thus emphasising the β -adrenoceptor nature of the cyclic AMP synthesising system. When the temperature was lowered to 24 °C, decreased effectiveness of isoproterenol and increased

activity of both adrenaline and noradrenaline in promoting cyclic AMP synthesis were observed. Furthermore, increases in cyclic AMP levels by isoproterenol and adrenaline were susceptible to blockade by the α -adrenoceptor blocker phentolamine.

At 37 °C cyclic GMP synthesis seemed to reflect the activity of the α -adrenoceptor as the order of potency of the catecholamines used was noradrenaline > adrenaline > isoproterenol (see also Fig. 1); and the effects, particularly at the low agonist concentrations, were susceptible to blockade by the α -adrenoceptor blocker phentolamine (see also Fig. 2). When the atria were cooled to 24 °C however, the reverse order of potency of the three catecholamines on cyclic GMP synthesis became apparent although the effects were again susceptible to blockade by phentolamine. In addition, the β -adrenergic blocking agent propranolol effectively blocked the cyclic GMP increases induced by all catecholamines at the colder temperature.

It seems therefore that the same set of β -receptors with its classical structural requirements and blocker susceptibility that trigger the synthesis of cyclic AMP at 37 °C, promoted the

Table 1 Effects of various concentrations of agonists alone and in the presence of antagonists on cyclic nucleotide levels in the rat atrium at 24 and 37 °C

		Cyclic AMP content (pmol per mg protein)						Cyclic GMP content (pmol per mg protein)					
		24 °C			37 °C			24 °C			37 °C		
		Alone	+ Pro- pranolol	+ Phen- tolamine	Alone	+ Pro- pranolol	+ Phen- tolamine	Alone	+ Pro- pranolol	+ Phen- tolamine	Alone	+ Pro- pranolol	+ Phen- tolamine
Isoproterenol	10 ⁻⁷ M	5.7 ± 0.4	10.1 ± 1.1†	9.5 ± 0.6†	6.7 ± 0.7	4.5 ± 0.6†	6.6 ± 0.3	2.30 ± 0.18	0.31 ± 0.01†	0.82 ± 0.32*	0.42 ± 0.03	0.23 ± 0.02*	0.36 ± 0.08
	10 ⁻⁶ M	18.3 ± 1.5†	—	—	19.6 ± 1.0†	—	—	0.65 ± 0.10*	—	—	0.50 ± 0.14	—	—
	10 ⁻⁵ M	22.3 ± 2.8†	12.9 ± 0.8†	15.6 ± 0.6†	37.1 ± 8.3*	13.4 ± 0.2†	20.7 ± 1.2	0.57 ± 0.07*	0.48 ± 0.02	1.19 ± 0.12‡	0.74 ± 0.04*	0.36 ± 0.08†	1.11 ± 0.19
Adrenaline	10 ⁻⁷ M	4.4 ± 0.3	8.9 ± 0.7†	9.3 ± 0.5†	4.0 ± 0.2	4.7 ± 0.6	7.8 ± 0.5†	1.34 ± 0.18	0.40 ± 0.10†	0.45 ± 0.05*	0.93 ± 0.02	0.26 ± 0.03*	0.59 ± 0.03*
	10 ⁻⁶ M	14.1 ± 0.4†	—	—	10.8 ± 1.3†	—	—	0.52 ± 0.06*	—	—	0.16 ± 0.05*	—	—
	10 ⁻⁵ M	16.8 ± 1.5†	11.9 ± 0.2‡	12.5 ± 0.6†	15.3 ± 0.4†	9.9 ± 0.5‡	12.5 ± 0.3‡	0.46 ± 0.03*	1.03 ± 0.16‡	0.63 ± 0.11	0.44 ± 0.03*	0.48 ± 0.06	1.10 ± 0.23
Noradrenaline	10 ⁻⁷ M	5.2 ± 0.3	10.2 ± 1.6*	9.0 ± 1.0†	3.3 ± 0.3	4.5 ± 0.5	8.9 ± 0.5†	0.92 ± 0.21	0.66 ± 0.12	0.25 ± 0.03*	1.09 ± 0.14	0.36 ± 0.05*	0.75 ± 0.10*
	10 ⁻⁶ M	13.4 ± 0.6†	—	—	8.7 ± 0.6†	—	—	0.39 ± 0.09	—	—	0.31 ± 0.06*	—	—
	10 ⁻⁵ M	17.3 ± 1.1†	13.7 ± 0.6†	17.5 ± 1.0	11.8 ± 0.9†	12.8 ± 1.0	14.0 ± 1.2	0.63 ± 0.05	0.42 ± 0.06	0.53 ± 0.07	0.20 ± 0.06*	0.85 ± 0.25†	0.73 ± 0.18‡

Experimental conditions and antagonist concentrations are the same as for Fig. 2. Results are means of six experiments ± s.e.m.

*Statistical significance from agonist alone at 10⁻⁷M, *P* < 0.05; †*P* > 0.01.

‡Statistical significance from agonist alone at 10⁻⁵M, *P* < 0.05; §*P* > 0.01.

synthesis of cyclic GMP at the colder temperature. Increased cyclic GMP synthesis at the colder temperature could not have resulted from the activation of new α -adrenoceptors that were dormant at 37 °C, as the order of potency noradrenaline > adrenaline > isoproterenol on cyclic GMP generation characteristic of classical α -adrenoceptors did not prevail. Increased cyclic GMP synthesis triggered by the β -adrenoceptor at the colder temperature may be regarded as either a change in substrate specificity of the catalytic subunit of the adenylyl cyclase enzyme from ATP to GTP with no change in the discriminator or receptor subunit¹⁸; or the coupling of the β -adrenoceptor subunit at the cold temperature with the catalytic subunit of guanylyl cyclase. A model conforming with

this latter possibility has already been proposed¹⁹. The continued susceptibility of cyclic GMP synthesis at the low temperature to the blocking effects of phentolamine indicates that this blocker must act as a cyclic GMP-generating site located past the apparently unchanged receptor, as has already been suggested²⁰. Propranolol, on the other hand, seems to act at the level of the receptor subunit itself.

At the colder temperature and low agonist concentrations, the observed propranolol-resistant increases in cyclic AMP in the presence of the β -adrenergic blocking agent could be secondary to blockade of the increases in cyclic GMP (Fig. 2). This may also explain cyclic AMP increases in the presence of phentolamine at both 37 and 24 °C, as both cyclic nucleotides

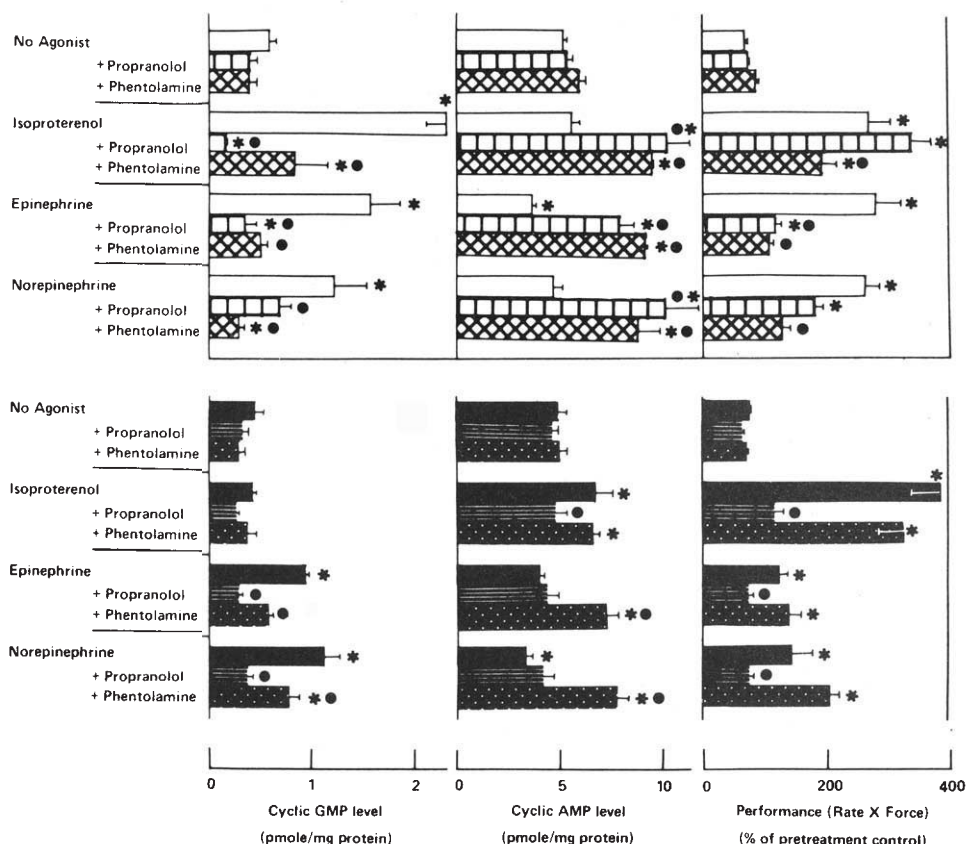


Fig. 2 Effects of various adrenergic agonists and antagonists on performance, cyclic AMP and cyclic GMP levels in spontaneously beating rat atria at 37 °C (a) and 24 °C (b). Experimental conditions are the same as for Fig. 1. Column heights represent the averages of 4–6 atria ± s.e.m. *Statistically significant differences (*P* < 0.05) from control (no agonist) at the same temperature; • statistically different from agonist alone at the same temperature. Isoproterenol, adrenaline, noradrenaline and propranolol were added in a final concentration of 10⁻⁷ M whereas the final concentration of phentolamine was 10⁻⁶ M. Propranolol and phentolamine were added to the bath 30 min before and the atria were quickly frozen 10 s after addition of catecholamines. Mean (± s.e.) heart rates during the control (predrug) period were 292 ± 6.4 and 105 ± 8.5 beats min⁻¹ at 37 and 24 °C, respectively. Basal contractile forces were 0.29 ± 0.03 g at 37 °C and 0.79 ± 0.08 g at 24 °C.

are known to influence the rate of hydrolysis of one another¹⁸. The reverse situation may exist in the case of propranolol at the higher temperature, that is, blockade of the changes in cyclic AMP levels induced by the catecholamines may be responsible for the effects of propranolol on cyclic GMP levels. It is unlikely that the effects of these catecholamines on cyclic nucleotide levels involve direct action on phosphodiesterase enzymes²¹. It is not clear from the present study which cyclic nucleotide has the major role of translating the effects of catecholamines into mechanical effects in the heart, particularly in the crucial low temperature-low concentration conditions. The data presented here indicate a possible antagonistic role for cyclic GMP to that of cyclic AMP, a role that seems less prominent at the lower temperature. This is supported by the observation that cyclic GMP seems to mediate the negative chrono- and inotropic effects of acetylcholine on the heart¹⁹.

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Two mechanisms for poststimulus hyperpolarisations in cultured mammalian neurones

Long lasting hyperpolarising responses following high frequency spike discharges in neurones from several sub-mammalian species have been attributed to either electrogenic ion transport^{1,2} or to a long lasting increase in K⁺ conductance^{3,4}. These prolonged hyperpolarising responses of whatever mechanism result in alterations of neuronal behaviour which may have important functional consequences for the integrative activity of the central nervous system (CNS). Similar potentials have been reported in studies on the intact mammalian CNS⁵⁻⁷, but the complexity of these preparations has prevented a thorough analysis. Such phenomena can be studied using intracellular recording techniques in dissociated cell cultures of mammalian neural tissue, and here we demonstrate post-stimulus hyperpolarising responses in cultured spinal neurones and suggest the underlying mechanisms.

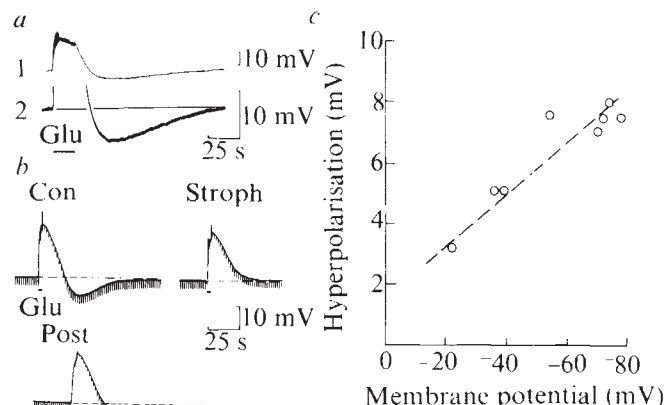
Spinal cords were dissected from 13-d-old mouse embryos, mechanically dissociated and plated at a density of about 10⁶ cells per dish (35 mm diameter, Falcon). After a brief exposure to a metabolic inhibitor (to suppress the rapid division of fibroblasts) cells were allowed to mature for a period of about 3 weeks. Cells were maintained in modified Eagle's medium with 10% horse serum added as described previously⁸. Experiments were carried out by placing a culture dish in a chamber on the modified stage of an inverted phase contrast microscope. Mature cultures contained two easily distinguishable populations of large neurones (25-50 μ m): dorsal root ganglion (DRG)

cells and multipolar spinal cord (SC) cells. These cells exhibited a full range of characteristic neuronal electrophysiology including membrane potentials of -40 to -60 mV, spike electrogenesis, and in the case of SC cells, synaptic transmission, as well as sensitivity to a number of putative transmitters^{8,9}. Neuronal somata were impaled under direct vision with 4 M K⁺ acetate-filled pipettes (20-40 M Ω). At the time of study MgSO₄ was usually added to a final concentration of 10 mM to suppress spontaneous synaptic activity. Standard electrophysiological techniques were used for intracellular recording and iontophoresis. Glutamate (0.6 M, pH 8) was iontophoresed on to cell somata from fine-tipped pipettes. Strophanthidin (3×10^{-4} - 7×10^{-4} M, Sigma), dissolved in medium, was leaked on to cells using coarse pipettes (tip diameter 25-50 μ m).

Glutamate, a putative excitatory transmitter in the mammalian CNS¹⁰, caused brisk depolarisation, usually evoking action potentials in over 95% of SC cells. Short pulses of glutamate (that is <1 s duration) elicited responses with roughly exponential falling phases. Longer lasting pulses evoked a more complicated sequence of events (Fig. 1a). During the drug application the cells depolarised to a steady plateau level without evidence of desensitisation. Following termination of the iontophoretic current, the membrane potential returned past the prestimulus level resulting in a long lasting (20-200 s) hyperpolarisation of variable magnitude (1-20 mV). The magnitude and duration were related directly to the duration and intensity of the glutamate application. This post-glutamate hyperpolarisation (PGH) was not associated with a change in input resistance (Fig. 1b) and did not have an apparent reversal potential. Conditioning hyperpolarisation of the membrane potential (through sustained injection of current) enhanced the PGH response in seven cells tested (Fig. 1c). PGH was present at potentials at which the hyperpolarising after-potential of the spike had reversed polarity, implying that the PGH could drive the membrane potential more negative than the K⁺ equilibrium potential.

PGH was rapidly and reversibly abolished by the local application of strophanthidin in six cells tested (Fig. 1b). In four cells application of the drug during PGH caused an

Fig. 1 Characteristics of PGH. *a*, Response of multipolar spinal cord cell to prolonged glutamate application (50 nA, shown by bar). Trace 2 is a high gain record of trace 1. Membrane potential -38 mV. *b*, Effect of strophanthidin on PGH. Constant applications of glutamate (34 nA, 8 s) were given before (con), during (stroph), and 5 min after (post) the local application of strophanthidin (3×10^{-4} M) to the cell soma. Downward deflections are voltage responses to injection of constant hyperpolarising current pulses (0.3 nA for con and stroph, 0.5 nA for post record). Input resistance does not change during PGH. The post PGH is longer lasting and smaller than the con PGH probably as a result of a reduction in peak pump rate caused by residual strophanthidin action. Membrane potential -65 mV. *c*, Relationship between PGH amplitude and membrane potential. Constant glutamate applications (50 nA, 12 s) were applied while the membrane potential was varied by passing steady polarising current through the recording electrode. Membrane potential at rest -38 mV.



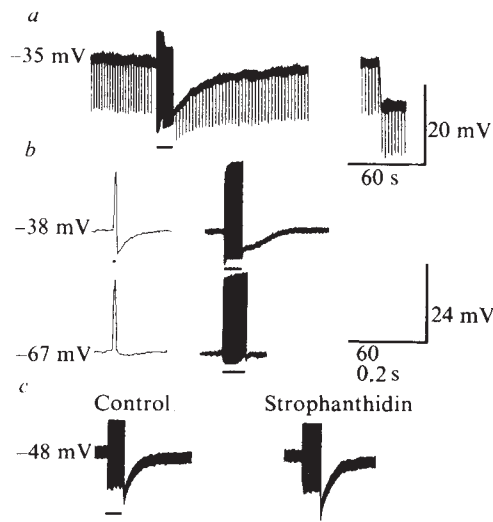


Fig. 2 Characteristics of PTH in DRG cells. *a*, Trains of depolarising current pulses (30 s^{-1}) elicited a spike train of same frequency (shown by bar), following which a slowly relaxing hyperpolarisation is seen. Downward deflections are voltage responses to the injection of constant current pulses (0.2 nA). Note the decrease in input resistance during PTH. Right-hand record in *a* shows that no change in resistance occurs when the membrane potential of this cell is passively moved in the hyperpolarising direction by steady current injection. *b*, Relationship between spike after-potential, PTH and membrane potential. After-potential following an action potential (induced by a single depolarising current pulse) and PTH elicited in the same cell by high frequency pulses (50 s^{-1}) are compared at different membrane potentials achieved by polarising current. *c*, Locally applied strophanthidin ($7 \times 10^{-4}\text{ M}$) does not alter PTH (50 s^{-1}). Calibration: 20 mV and 60 s (*a* and *c*); 0.2 s (*b*, left).

immediate depolarisation averaging 15 mV. (Strophanthidin application to cells not undergoing PGH led to rapid, reversible, depolarisation averaging $5 \pm 2\text{ mV}$ (s.d.; $n = 12$).) Drug application did not cause any detectable changes in input resistance, nor did it interfere with the depolarisation elicited by glutamate.

DRG cells, which are unresponsive to glutamate (B.R.R. and P.G.N., unpublished), also exhibited a form of poststimulus hyperpolarisation. In these cells high frequency spike trains (elicited by depolarising current pulses) were followed by hyperpolarisations lasting several seconds (Fig. 2*a*). Although this post-tetanic hyperpolarisation (PTH) is similar to the hyperpolarising responses in SC cells with regard to magnitude and duration, PTH has several distinguishing characteristics. During PTH, input resistance was decreased, returning to its control value as the membrane potential returned to the baseline level (Fig. 2*a*). PTH magnitude was dependent on membrane potential; its reversal potential was identical to that of the spike after-potential (Fig. 2*b*). Strophanthidin, applied to DRG cells, did not alter PTH (Fig. 2*c*).

PTH in DRG cells seems to result from a prolonged period of increased K^+ conductance. This is suggested by the conductance increase during PTH, the reversal potential of PTH being coincident with the reversal potential of the spike after-potential, and the insensitivity of PTH to strophanthidin. In invertebrate cells a response with similar characteristics follows spike trains and has been shown to depend on Ca^{2+} influx during action potentials with a subsequent increase in K^+ conductance^{3,4}. As the DRG cell has a Ca^{2+} component to its spike (J. Fischbach and M. Dichter, unpublished), it is possible that PTH may result from a similar sequence of events.

PGH in SC cells fulfils many of the electrophysiological criteria which identify potentials produced by electrogenic transport²; it occurs without change in membrane conductance, can exceed the K^+ equilibrium potential (as reflected by the reversal potential of the spike after-hyperpolarisation), and is abolished by the glycoside strophanthidin at a concentration known to inhibit the Na^+ pump. As the glutamate response has

an equilibrium potential around -20 mV , it is likely that the response involves a substantial increase in Na^+ permeability (ref. 11 and N. Brookes, unpublished). Prolonged glutamate application therefore would lead to an increase in intracellular Na^+ concentration, causing an increase in the rate of Na^+ pumping¹². In fact, spike amplitude is depressed during PGH, suggesting that the Na^+ gradient across the membrane is reduced (not illustrated). Other experiments to be reported in detail elsewhere show that Na^+ injection or tetanic spike discharge in SC cells can result in hyperpolarisations similar to PGH. Thus, we conclude that PGH as well as these other potentials results primarily from the current generated by increases in the rate of electrogenic Na^+ transport triggered by Na^+ loading induced by certain chemical and electrical stimuli. The increase in PGH which is seen with hyperpolarisation (Fig. 1*c*) presumably results from increased Na^+ loading that would be expected as a result of the increased Na^+ driving force produced by the hyperpolarisation. Our results also suggest that there is an electrogenic component to the resting potential in SC cells amounting to about -5 mV .

The significance of the potentials reported here is not well understood. Such long-lasting neural signals can have immediate effects on information processing^{13,14}, and may encode important and unique aspects of previous nervous activity. It is tempting to speculate that these or similar processes may intervene between rapid electrochemical events and more lasting biochemical phenomena. An obvious opportunity for this sort of linkage exists in DRG cells in which both PTH and the initiation of axoplasmic transport¹⁵ may have an important dependency on Ca^{2+} . The PGH in SC cells, on the other hand, is clearly associated with an increase in Na^+ transport and thus ATPase activity. The attendant effects on energy metabolism might be expected to have a variety of biochemical sequelae.

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Activation of a voltage-insensitive conductance by inward calcium current

ELECTRICAL excitability is more complex in cell bodies than in axons. In various nerve cells there is a voltage-sensitive permeability to calcium as well as to sodium and potassium. Furthermore, increased calcium in the cytoplasm of nerve cell bodies increases their potassium permeability. In voltage-clamped *Helix* neurones, Meech and Standen^{1,2} reported that a substantial fraction of the potassium conductance activated during a depolarisation depends on an inward calcium current.

We have studied the sensory epithelium of the electroreceptor (ampulla of Lorenzini) of the skate. In this epithelium, which

can generate all-or-none action potentials^{3,4} of 60 mV, we have found that the repolarising phase of the action potential results from a late outward current initiated when calcium ions enter the cytoplasm of the excitable cells. Because there is no detectable voltage-sensitive outward current, we conclude that a distinct calcium-activated conductance mechanism is involved.

Skate electroreceptor epithelium consists of a single layer of interspersed receptor and supporting cells (Fig. 1b). The epithelium forms a lobulated sac, or ampulla of Lorenzini, which is connected to an external orifice by a long canal (Fig.

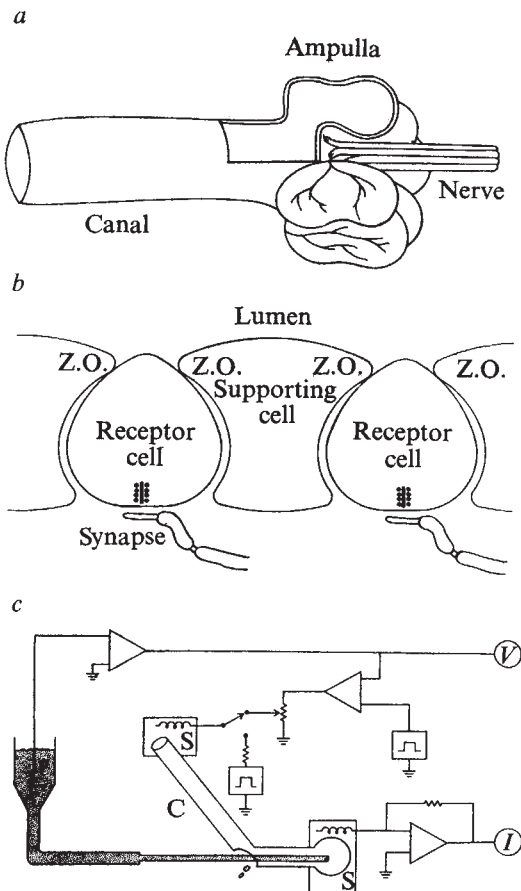


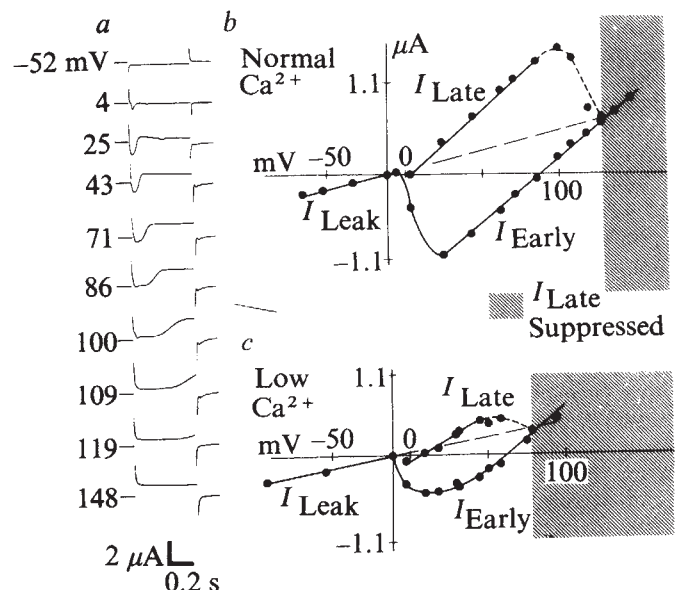
Fig. 1 *a*, Skate ampullary electroreceptor and canal (after Waltman⁵). *b*, Details of two receptor cells in the ampullary epithelium. Occluding junctions (zonulae occludentes, Z.O.) occur between receptor cells and adjacent supporting cells. The basal membranes form characteristic ribbon synapses with the afferent nerve fibres⁶. *c*, Experimental arrangement for current clamping, voltage clamping, and internally perfusing an ampulla. The ampulla with its canal (average diameter 1 mm, average length 7 cm) was removed from a skate (*Raja ocellata* or *R. erinacea*). The open end of the canal was placed in one saline pool (S) and the ampulla in another. Little current flows along the external surface of the canal (C) because it was rinsed with a solution of 526 mM sucrose and 350 mM urea before it was placed in the recording chamber. A 100- μ m glass cannula was introduced through a nick in the canal wall. The cannulated portion of the canal was in a Vaseline gap. The uncannulated portion was in air. The saline pools contained cerebrospinal fluid from the cranial cavity or occasionally Fühner's⁹ elasmobranch saline. The perfusate (P) was under slight pressure. Effluent was aspirated. All perfusates were made from a control solution containing 428 mM NaCl, 13 mM KCl, 50 mM MgCl₂, 10 mM CaCl₂, 75 mM urea and 5 mM HEPES at pH 8.1. Ionic exchange inside the ampulla was limited by diffusion through the gelatinous material that fills the individual lobules. When the ampulla was perfused with phenol red, noticeable diffusion into all the lobules required about 15 min. Epithelial current was measured by a current-voltage transducer with output *I*. Epithelial voltage was measured at the perfusion cannula, and controlled using an operational amplifier and a voltage source. Constant current pulses were applied by connecting the electrode at the canal orifice to a second voltage source through a $10^8 \Omega$ resistor.

1a). The canal is a core conductor, the wall of which is electrically linear and has a high resistivity⁵. All cell surfaces abutting on the lumen of the ampulla and its canal are joined by occluding junctions which presumably prevent extracellular leakage of current or ions across the epithelium. The occluding junctions separate the excitable luminal faces of the receptor cells from their basal faces, which are innervated by afferent fibres of the VIIIth cranial nerve (Fig. 1b).

The electroreceptor epithelium was voltage clamped as shown in Fig. 1c. During lumen-positive voltage steps (which hyperpolarise the luminal membranes), epithelial current was constant after the capacitive transient. When the lumen was made negative (depolarising the luminal membranes), however, active currents were observed (Fig. 2a). Excitatory (lumen-negative) voltage steps of moderate amplitude evoked an early current which flowed inward across the luminal membranes followed by a maintained outward current. At voltages less than 30 mV, multiple peaks of inward current sometimes occurred. We attribute active currents to the receptor cells because of the associated postsynaptic potentials that could be recorded in the afferent nerve fibres. The conductance changes responsible for the active currents are attributed to the luminal faces of the receptor cells because these currents are dramatically altered by ionic substitutions in the lumen (see below), but are only slightly affected by drastic alterations outside the basal membranes, for example, chelation of calcium with EGTA, or 90% replacement of NaCl with potassium acetate.

When increasingly large excitatory voltage steps were applied across the epithelium, onset of the late outward current became slowed and delayed (Fig. 2a). At about 130 mV lumen-negative, activation of the late outward current was totally suppressed. The remaining early current did not inactivate significantly during the clamping pulse. A current-voltage relationship of the electroreceptor epithelium is shown in Fig. 2b. Excitatory (lumen-negative) voltages are to the right of the vertical axis. For excitatory voltage steps, the peak inward and late outward

Fig. 2 *a*, Currents recorded from skate electroreceptor epithelium during perfusion with the control solution. Current flowing inward across the luminal membranes of the receptor cells and outward across the basal membranes is defined as inward current and shown downward. Voltage displacements that depolarise the luminal membranes (lumen-negative) are defined as positive. *b*, A current-voltage relationship plotted from the data in *a*. The vertical axis is placed at the holding potential, which is the potential developed by the epithelium when no current is allowed to flow in the canal. The reversal potential for the early current, obtained by extrapolation of the leakage current (long dashes), is also the potential at which the late outward current is suppressed. *c*, Current-voltage relationships obtained from the same preparation after the free calcium concentration in the lumen had been reduced by perfusion with EGTA.



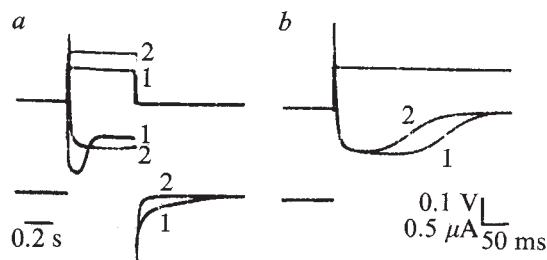


Fig. 3 *a*, Repolarisation tail currents recorded from skate electroreceptor epithelium. Two sweeps with voltage steps of different amplitude are superimposed. The smaller stimulus, 1, evokes a late outward current and a repolarisation tail current lasting 600 ms. The larger stimulus, 2, exceeds the reversal potential for the early current, and evokes no late current and no prolonged repolarisation tail. *b*, Facilitated onset of the late outward current by a conditioning stimulus. Two uniform voltage steps are applied 2 s apart and the currents are superimposed. The early current is identical for the two stimuli, but the late outward current evoked by the conditioned stimulus, 2, occurs at much shorter latency than that evoked by the conditioning stimulus, 1.

currents were linear functions of voltage over a broad range. For lumen-positive steps, current was also linearly related to voltage, the slope of the line being the leakage resistance.

We were able to demonstrate that the resistance immediately after the onset of an excitatory voltage displacement (instantaneous resistance) was equal to the leakage resistance. (This demonstration was possible because the form of the capacitive transient did not depend on the absolute voltage or the epithelial conductance. Capacitive transients could therefore be subtracted from the total current.) Moreover, experiments with superimposed pulses showed that the instantaneous resistance of the epithelium during maintained early and late currents was equal to the corresponding slope resistance. Knowing these instantaneous resistances, it is possible to determine the reversal potential for the early current. This reversal potential, about 130 mV, was equal to the suppression potential for the late outward current.

Early current in skate electroreceptors is carried by calcium as shown by the following observations: (1) The action potential was unaffected when the lumen of the ampulla was perfused with 0.1 mM TTX. (2) The action potential was abolished by perfusion with cobalt. (100 mM CoCl₂ was prepared by isosmotic substitution for NaCl in the control saline. Because of diffusion delays, the effective concentration at the ampullary epithelium must have been substantially lower.) (3) Inward current was abolished by a zero calcium solution containing EGTA. (20 mM EGTA and 60 mM HEPES were brought to pH 8.1 with NaOH and isosmotically substituted for NaCl.)

The current-voltage relationship of Fig. 2c was obtained during perfusion with a solution containing normal (10 mM) Ca, 20 mM EGTA and 60 mM HEPES. The data were taken after about 15 min, before ionic equilibration was complete. Although the free calcium concentration is not known, the reversal potential for the early current was clearly reduced, as was the magnitude of the early current at all voltages. Significantly, the suppression potential for the late outward current still corresponded to the reversal potential for the early current. These observations suggest that the early current is carried by calcium, and that the late current is not activated unless there is a net influx of calcium into the cytoplasm. This interpretation is corroborated by the fact that no delayed rectification was seen when the epithelial action potential was abolished by perfusion of the luminal membranes with EGTA or cobalt.

When the epithelium is repolarised following activation of the late outward current, a tail current lasting about 600 ms is observed (Fig. 3a, trace 1). Prolonged tail currents are not observed when the excitatory voltage stimulus exceeds the reversal potential for the early current and there is no late current (trace 2). Prolonged tail currents are augmented, but

cannot be shortened by repolarising the epithelium to increasingly lumen-positive voltages. These observations suggest that the prolonged tail current flows in a voltage-insensitive conductance that is activated by an early inward current.

The absence of any voltage-sensitive component of the slow current requires that a different type of conductance mechanism, presumably a distinct macromolecule, be responsible for terminating the action potential in skate electroreceptors. An important property of this calcium-activated conductance is that it undergoes facilitation. In Fig. 3b, two identical pulses were applied 2 s apart. The conductance of the epithelium returned to normal between pulses and the size of the inward current evoked by the two pulses was uniform. The onset of the late outward current, however, occurred considerably earlier during the second stimulus. This effect of a conditioning stimulus lasted about 10 s. It was not observed when the conditioning stimulus exceeded the reversal potential for the early current. Residual calcium may therefore have been involved. Facilitated onset of a calcium-activated hyperpolarising conductance may mediate sensory accommodation in skate electroreceptors⁷.

Evidence for a calcium-activated hyperpolarising conductance has been adduced for each of several nerve cell bodies that have been studied in this respect^{6,8}. It seems likely that the underlying molecular basis is the same in these cells as in skate electroreceptors, although in neurones experiments are complicated by the presence of a voltage-sensitive late conductance. Calcium-activated conductances could have a major role in the regulation of electrical activity in cell bodies, particularly if they exhibit the facilitated onset we have described in skate electroreceptors.

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Selective elimination of taste responses to sugars by proteolytic enzymes

INTERACTION between taste stimuli and taste receptors has been suggested as the initial event preceding the taste cell excitation, and, based on a number of biochemical investigations, the receptors are presumed to be proteinaceous in nature¹⁻³. For example, a protein which combines with sweet substances was extracted from bovine⁴ and rat tongues⁵. Direct evidence for the ability of the extracted protein to bind with sugars has come from experiments using ¹⁴C-labelled fructose and polyacrylamide gel electrophoresis or Sephadex gel filtration^{6,7}. The presence of a sugar-binding receptor in taste buds has also been indicated by an experiment in which suspensions of papillae containing taste buds were found to bind more ¹⁴C-sucrose than those without taste buds. Furthermore this binding ability could be abolished by heating⁸. Therefore, if taste receptors for sweet substances are composed of a protein, the neural responses to sugars would be eliminated after digestion of the receptor protein by proteolytic enzymes. To check the above hypothesis, various proteases were applied to the rat tongue surface and impulses from the taste nerve were recorded. In addition,

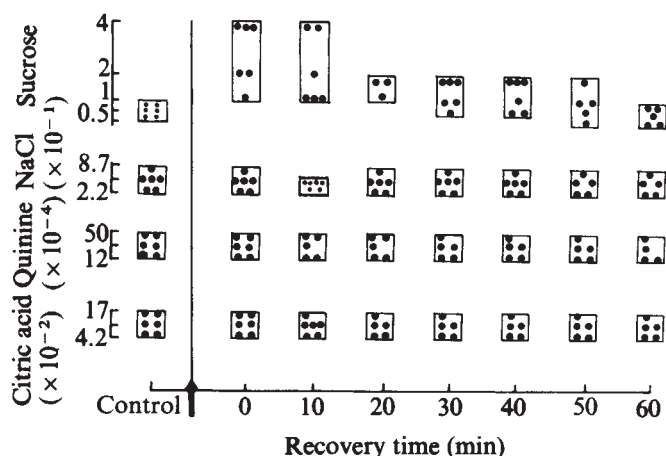


Fig. 1 Integrated responses of rat chorda tympani to stimulation of the tongue with 1 M sucrose, 0.02 M quinine-HCl, 0.01 M HCl, and 0.05 M NaCl before (control) and after treatment with 2% pronase E for 30 min. The two right-hand columns indicate the responses after rinsing the tongue with water. Female Sprague-Dawley rats (150–250 g) were anaesthetised by intraperitoneal injection of sodium amobarbital (70 mg per kg body weight). Integrated responses to taste stimuli were recorded from the chorda tympani⁹. In each experiment, the integrated responses to four basic taste stimuli (0.05 M NaCl, 0.01 N HCl, 0.02 M quinine hydrochloride and 1.0 M sucrose) were recorded first as controls. Subsequently, one of the enzymes (with pH adjusted with phosphate buffer solutions at 30–50 °C) was applied to the rat tongue through a glass flow-chamber for 20 min or more, after which the tongue was rinsed with 300–400 ml tapwater and the above series of stimulations repeated. Commercially obtained enzymes were used without further purification.

an alteration of the taste threshold produced by the enzymes were also examined in human subjects.

Application of various proteolytic enzymes such as trypsin (pH 7.0), papain (pH 7.0) and neutral (pH 7.0) and acidic (pH 4.7) proteases to the tongue surface for more than an hour did not produce any effect on the neural responses to the four basic taste stimuli (Fig. 2). On the other hand, when Pronase E (pH 7.0) or semi-alkaline protease (pH 8.0) was applied to the tongue for 20 min, the chorda tympani response to 1 M sucrose was suppressed (Figs 1 and 2), whereas responses to the other three taste stimuli were hardly affected (Fig. 1). Similar suppression of responses to various sweet substances was also observed. Responses to 1 M glucose, 1 M fructose, 0.8 M sorbitol

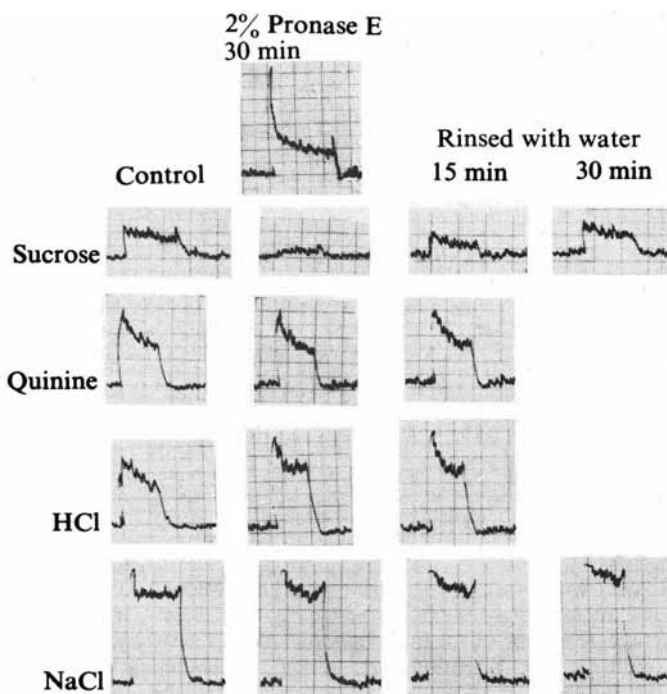


Fig. 3 Changes in taste thresholds in human subjects by application of Pronase E (2%, 10 min, at arrow). Each box indicates the range of the taste threshold concentration (%) for sucrose, NaCl, quinine-HCl and citric acid before and after application of Pronase. Note the recovery to normal of the elevated threshold for sucrose. For sensory examinations a filter paper immersed in Pronase solution at the same concentration as that applied to the rat tongue was placed on the tongue of human subjects for a period of 10 min. After the filter paper had been taken off, the tongue was rinsed with distilled water, and taste solution applied with a pipette to the same area. The tongue was rinsed with distilled water after each stimulation with a taste solution.

and even 0.005 M saccharin were completely eliminated by the previous application of Pronase E or semi-alkaline protease, and responses to 0.8 M glycine and 0.8 M DL-alanine, which produce sweet taste in man, were also depressed markedly. By rinsing the tongue with water for a period of 30 min or more after the enzyme application, the eliminated responses to the sweet substances were completely recovered to the same magnitude as found for controls (Figs 1 and 2). In contrast, Pronase E, which was

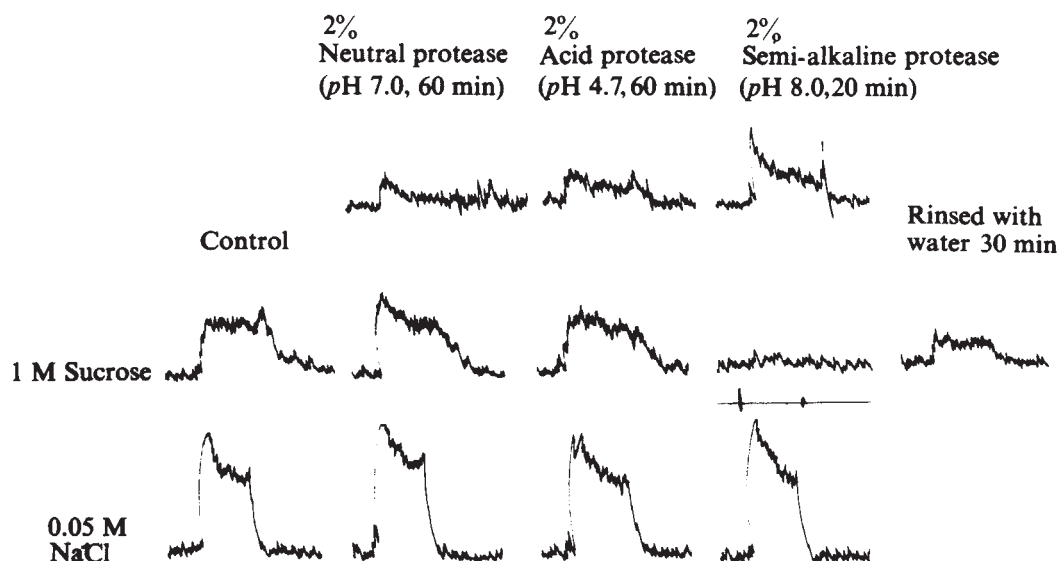


Fig. 2 Integrated responses of the rat chorda tympani to 1 M sucrose and 0.05 M NaCl (left column). The three middle columns indicate the responses after treatment with 2% neutral, acid and semi-alkaline proteases and the right column shows those after rinsing the tongue with water. On-off markers in sucrose response indicate the period of application of taste solution to the tongue.

inactivated by heating at 80 °C for 5 or 10 min, produced little effect on the integrated response to sucrose.

Essentially the same effect for Pronase as that obtained by the neural experiments was demonstrated in the sweet sensation of man (Fig. 3). The mean ($\pm$ s.d.) threshold concentration for sucrose in man treated with Pronase was $2.8 \pm 1.3\%$ (w/v), which is about five times greater than normal ($0.58 \pm 0.32\%$), and the difference is significant ($0.01 > P > 0.001$; Student's *t* test). The elevated threshold reverted to normal by rinsing the tongue with water for 20–60 min. On the other hand, thresholds for the other three tastes remained within the normal range ($0.44 \pm 0.21\%$ for NaCl, $0.003 \pm 0.0013\%$ for quinine hydrochloride, and $0.099 \pm 0.053\%$ for citric acid) before and after application of Pronase (Fig. 3). The present sensory data confirmed the observations on the neural response in the rat.

In the present study it has been demonstrated that the proteolytic enzyme selectively suppresses rat chorda tympani responses to sweeteners without affecting those to other kinds of taste stimuli. An elevated threshold for sugar by application of the enzyme was also found in the sensory examinations. Thus, the neurophysiological experiments on rats qualitatively duplicate the sensory data in men. Both kinds of study may indicate that the sugar-binding ability of the receptor is depressed, possibly as a result of digestion of the receptor protein by the enzyme. Consequently, our findings support the conclusion, derived from biochemical investigations^{4–8}, that the sugar receptor molecules are composed of a protein. The recovery of taste responses after rinsing the proteolytic enzyme with water may be the result either of the strong repairing action of the protein or of a rapid turnover mechanism of the receptor protein synthesised in taste cells.

Giroux and Henkin¹⁰ have reported that proteolytic enzymes produce a non-selective increase in the threshold for each of the four basic tastes in man, and that alterations in taste acuties lasted for as long as 12 h after application of Pronase. Their results hardly agree with those from our experiments. This difference may be the result of a difference in the dosages used. In their examinations the total dosage of Pronase was 6 mg in a buffer solution, and the enzyme was thoroughly circulated within the oral cavity, which produced sensations of tingling and burning. In our sensory examinations the total dosage of Pronase absorbed in a filter paper placed on the tongue was about 1 mg. The actual concentration affecting taste buds may be less, because of dilution by saliva. This small dosage produced a light astringent taste and depressed the sugar response alone. Tingling and burning sensations after the enzyme treatment in the former experiments may suggest that not only the protein composing the sugar receptor but also the taste cell membrane responsible for other stimuli would be simultaneously digested by a strong proteolysis, leading to long lasting alteration in the thresholds not only for the sweet taste but also the other three tastes.

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Evidence for phosphofructokinase in chloroplasts

ATTEMPTS to demonstrate phosphofructokinase activity in chloroplasts have so far been unsuccessful^{1–4}, thus raising the question of whether the transitory starch formed in the chloroplast during photosynthesis can subsequently be metabolised to triose phosphates within the chloroplast. Such a conversion would seem beneficial if the products of starch degradation are required to move freely from the chloroplast to the cytoplasm, since triose phosphates, but not hexose phosphates, are actively and rapidly transported across the chloroplast membrane⁵. The apparent absence of phosphofructokinase from chloroplasts was also discouraging since this enzyme is considered one of the principal regulatory enzymes of carbohydrate metabolism⁶ and the regulatory properties of the plant enzyme have been shown consistent with the control of starch metabolism⁷. Investigations in this laboratory have now demonstrated that chloroplasts do contain phosphofructokinase activity.

Spinach chloroplasts were isolated by homogenising 24 g leaves for 5 s in 120 ml of the isolation medium described by Lilley *et al.* (ref. 8). The homogenate was filtered through Miracloth, centrifuged at 2,500*g* for 80 s and the pellets of chloroplasts broken in 30 ml of a solution of pH 7.7 consisting of 5 mM MgCl₂, 5 mM dithioerythritol and 1 mM EDTA and containing 1 g Polyclar AT. A sample of the supernatant was collected as representative of the cytoplasmic fraction; dithioerythritol was added to 5 mM, the pH adjusted to 7.7 and 1 g Polyclar AT stirred in. After 10 min these chloroplast and cytoplasmic samples were centrifuged at 22,000*g* for 20 min, the supernatants collected and that from the chloroplasts was concentrated through a Diaflo XM-50 filter to 4 ml. Samples (2 ml) of each of the concentrated chloroplast extract and cytoplasmic fraction were dialysed for 3 h against 1 l of a solution of pH 7.7 containing 5 mM imidazole-HCl, 1 mM MgCl₂, 1 mM dithioerythritol and 0.5 mM EDTA.

The activity of phosphofructokinase is compared in Table 1 with the activities of three marker enzymes: non-reversible glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is restricted to the cytoplasm⁹, the NADP-linked reversible GAPDH found only in the chloroplast², and fructose-1,6-diphosphate aldolase (FDP-aldolase) which occurs both in chloroplasts and the cytoplasm². The specific activity of non-reversible GAPDH in the chloroplast extract was only 2% of that in the cytoplasmic fraction, indicating that cytoplasmic contamination of these chloroplasts isolated in aqueous media was minimal. In contrast, considerable phosphofructokinase activity was detected in the chloroplast extract, the specific activity being 25% of that in the cytoplasmic fraction. The clear difference between the ratios of the specific activities of these two enzymes (Table 1) provides strong evidence that the phosphofructokinase activity detected in the chloroplast extract did not originate from cytoplasmic contamination of chloroplasts during isolation but rather represents an authentic localisation of phosphofructokinase with the chloroplast.

The activities of chloroplast GAPDH and FDP-aldolase (Table 1) show that a considerable number of the chloroplasts were broken during isolation; assuming all the chloroplast GAPDH activity and 65% of the FDP-aldolase activity are located in chloroplasts² the results in Table 1 show that enzyme loss from chloroplasts was between 65 and 80%. Consequently the total chloroplast phosphofructokinase activity shown in Table 1 is probably only a fraction of the true value.

Several lines of evidence confirm the presence of phosphofructokinase activity. The enzyme activity was dependent on both fructose-6-phosphate and ATP; no NADH oxidation was observed in the absence of either substrate if the preparation was dialysed before assay. Both phosphoenolpyruvate and

Table 1 Activities of phosphofructokinase and three marker enzymes in a chloroplast extract and cytoplasmic fraction from spinach leaves

	Chloroplast extract Total activity*	Specific activity†	Cytoplasmic fraction Total activity*	Specific activity†	Ratio: s.a. chloroplast s.a. cytoplasm
Phosphofructokinase	1.9	0.25	31	1.0	0.25
Non-reversible GAPDH	0.40	0.05	72	2.4	0.02
Chloroplast GAPDH	320	45	1200	39	1.1
FDP-aldolase	110	14	310	10	1.4

* Measured as $\mu\text{mol per h per } 10 \text{ g fresh weight of leaves}$.† Expressed as $\mu\text{mol per mg protein per h}$.

Enzyme activities were determined at 22 °C. The reaction mixtures for phosphofructokinase contained, in a final volume of 1 ml, 50 μmol imidazole-HCl buffer (pH 7.7), 2.5 μmol MgCl_2 , 2.5 μmol dithioerythritol, 1.5 μmol D-fructose-6-phosphate, 0.5 μmol ATP, 0.08 μmol NADH, 1 unit aldolase, 12 units triosephosphate isomerase, 1 unit α -glycerophosphate dehydrogenase and 50–100 μl extract. The composition of reaction mixtures for the determination of FDP-aldolase and chloroplast GAPDH activities were based on those described earlier²; the buffers used in the present experiment were imidazole-HCl (pH 7.7) and triethanolamine-NaOH (pH 8.0) respectively. Non-reversible GAPDH was assayed as described previously for cell-free plant extracts³. All assay enzymes were dialysed before use to remove $(\text{NH}_4)_2\text{SO}_4$. Activities were calculated from changes in extinction of reaction mixtures at 340 nm measured spectrophotometrically. Protein was determined by the Biuret procedure as described previously⁴.

relatively high concentrations of ATP inhibited the chloroplast phosphofructokinase activity: phosphoenolpyruvate at 0.1 mM produced more than 70% inhibition, while the activity with 4.8 mM ATP was less than half that with the optimum ATP concentration of 0.1 mM. Inhibition with increased levels of ATP is a common property of phosphofructokinase⁶ and sensitivity to inhibition by phosphoenolpyruvate is a notable feature of plant phosphofructokinase⁷. Some of these regulatory phenomena may explain why phosphofructokinase activity was not found in chloroplasts in earlier studies^{1–4} particularly if ATP levels used in those assays were too high.

Preliminary experiments in which intact chloroplasts were added to a solution containing the substrates for phosphofructokinase showed that the product fructose-1,6-diphosphate was not formed when the solution was isotonic (containing 0.33 M sorbitol) and the chloroplasts remained intact, but product was formed at a rate of approximately 2 $\mu\text{mol per mg chlorophyll per h}$ when sorbitol was omitted and the chloroplasts were broken. These results not only confirmed that the phosphofructokinase was contained within the chloroplast, but also demonstrated an activity compatible with the amount of starch expected to be metabolised in chloroplasts. It is not inconceivable that chloroplast phosphofructokinase catalyses a regulatory step in the degradation of starch in the chloroplast. Efforts are now being made to separate and partially purify the chloroplast and cytoplasmic phosphofructokinases from leaves with a view to relating their regulatory properties to the carbon metabolism of photosynthesis. Initial results are encouraging.

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collagenous protein can be solubilised in conditions of neutral pH but the collagen remains insoluble. Extracts of decalcified bone prepared in this manner contain small amounts of plasma proteins which derive in part from the presence of blood vessels within the tissue^{1,2}. There is evidence, however, that some plasma proteins, including albumin, form an integral part of the calcified matrix³. When ¹⁴C-glucosamine or ¹⁴C-glucosamine-labelled rabbit total plasma protein is injected into a rabbit, some activity is incorporated into the bone⁴. Using the procedure of Herring⁵ to separate the EDTA-soluble proteins of the bone matrix, most of the activity was shown to be present in the less acidic glycoprotein fraction, much of this in one glycoprotein which was also present as a minor component of blood⁴. This G2B glycoprotein had the electrophoretic mobility of an α globulin and a molecular weight by SDS gel electrophoresis⁶ of about 50,000. Studies by Ashton *et al.* (private communication) support the view that the G2B glycoprotein they have investigated in rabbit⁴ and bovine bone⁶ is the analogue of human α_2 HS glycoprotein.

In this report we provide evidence by immunoprecipitation and passive haemagglutination techniques that α_2 HS glycoprotein (an α globulin of estimated molecular weight 49,000 by ultracentrifugal analysis¹⁰ present in human plasma at a concentration of about 60 mg dl⁻¹) is concentrated extracellularly in the matrix of normal human bone and can be located in areas of mineralisation in bone using an immunofluorescent antibody-staining technique.

The non-collagenous proteins of normal adult human cortical bone were isolated by sequential extraction of the powdered bone with 0.5 M EDTA at pH 7.6. The concentrated extract, dialysed free of mineral salts, and human plasma each gave a single precipitin line of complete identity when they were diffused against the antiserum to plasma α_2 HS glycoprotein. The antiserum used in these studies was raised in rabbits against human plasma α_2 HS glycoprotein and was specific for the latter, as judged by immunoelectrophoresis and double diffusion against plasma proteins. Tanned sheep erythrocytes, coated with the non-collagenous bone protein as described previously¹, agglutinated in the presence of antiserum to α_2 HS glycoprotein but not with antisera to two other human plasma α globulins, α_1 antitrypsin and α_2 macroglobulin (Table 1). The latter two proteins are present in plasma at concentrations approximately three times higher than α_2 HS glycoprotein in normal individuals. This suggested that the identification of only the latter protein in bone in these conditions did not derive from the presence of blood within the tissue. The antisera to the three α globulins were each adsorbed with lyophilised bone protein and then incubated with human plasma protein-coated tanned sheep erythrocytes. These preparations had negligible effect (Table 1) on the haemagglutination titres of two of the antisera but the activity of that against α_2 HS glycoprotein was destroyed. By radial immunodiffusion in an agarose gel containing antibody, the concentration of α_2 HS

Localisation of plasma α_2 HS glycoprotein in mineralising human bone

THE protein of the organic matrix of bone consists mainly of collagen in association with a small proportion of non-collagenous proteins. When bone is decalcified, much of the non-

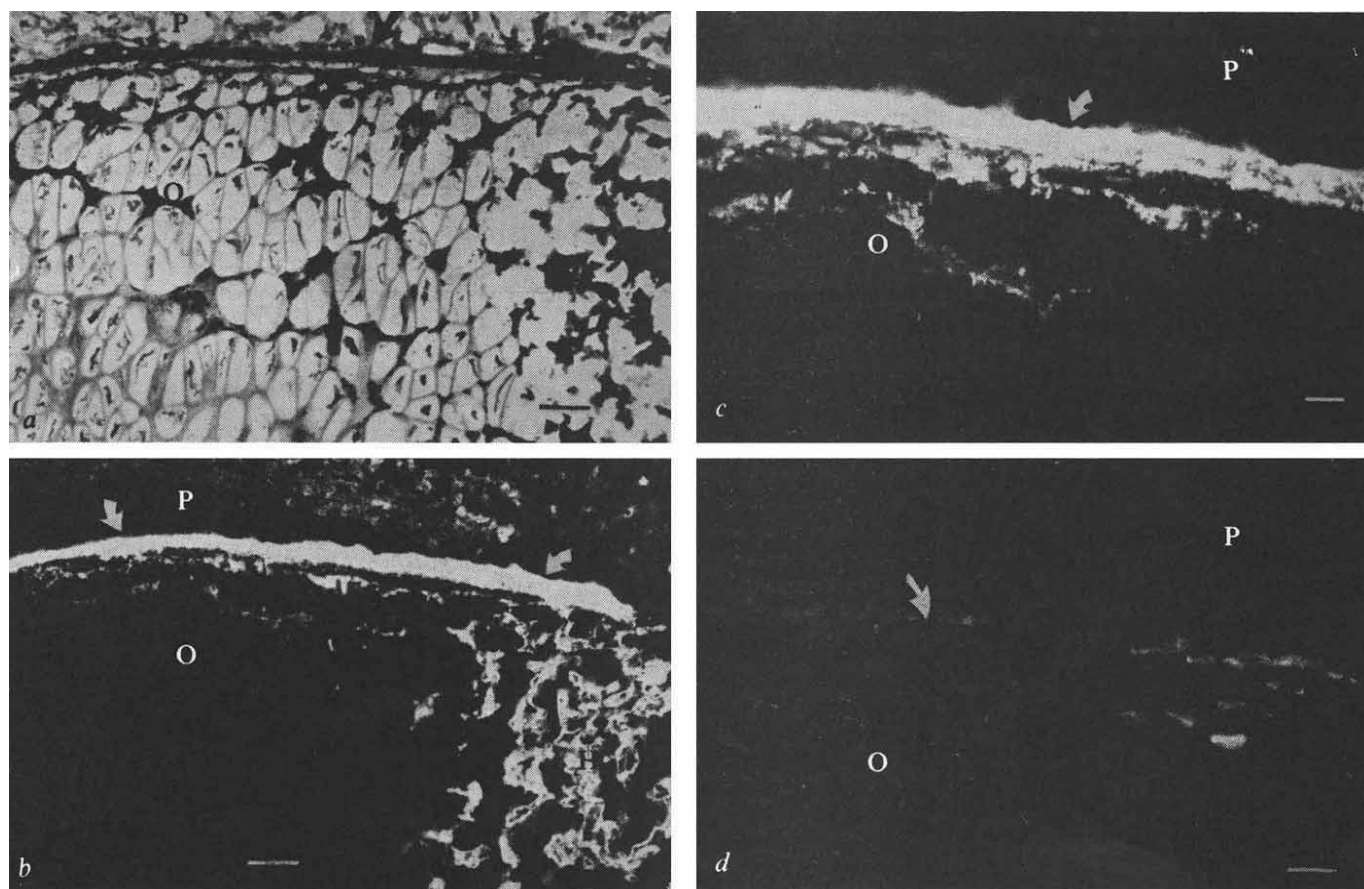


Fig. 1 Longitudinal 6 µm thick sections of femur. Frozen sections were fixed for 5 min in formaldehyde as described previously⁹. Sections were then washed in phosphate-buffered saline (PBS) for 30 min. *a*, Stained with von Kossa's reagent and counterstained with haematoxylin and chromotrope. A band of brown/black staining (arrows) in the periosteum (P) overlays staining for calcium in the hypertrophic cartilage which is either heavily ossified (H) or partially ossified (O). *b-d*, Immunohistochemical localisation of α_2 HS glycoprotein. Sites of binding of rabbit antibody Fab' to α_2 HS glycoprotein in tissue sections were detected by subsequently using fluorescein isothiocyanate-labelled pig antibody Fab' directed against rabbit Fab'. Thus sections were initially treated with pepsin digests (at 1.6 mg ml⁻¹) of 33% (NH₄)₂SO₄ immunoglobulin G-rich fractions isolated from the antiserum to α_2 HS glycoprotein (*b* and *c*) or non-immune rabbit serum (control); *d*, as described previously⁹. Sections were washed in PBS and finally stained with Fab' isolated from a pig antiserum to rabbit Fab', and labelled with fluorescein isothiocyanate at a concentration of 2 mg ml⁻¹. Sections were finally washed, mounted and examined (for details see ref. 9). *b* and *c*, Intense green extracellular fluorescence can be seen in the calcified band in the periosteum (arrows) and in the heavily ossified sites in the shaft (H) is magnified region of *b*. Less intense staining is present in sites of partial ossification (O). *d*, No intense green fluorescence was detected and only weak non-specific cellular staining was seen. Comparable sites are indicated as in *b* and *c*. Scales: *a* and *b*, 50 µm; *c* and *d*, 20 µm.

glycoprotein was estimated as 0.1% of the organic matrix of bone.

To establish whether the protein was incorporated into bone during the process of mineralisation, attempts were made to localise the protein by immunohistochemical techniques. The tissue used was the femur of a human foetus aged approximately 12 weeks (obtained after a therapeutic abortion). The femur was placed in a solution of 7% gelatin and 0.09% NaCl at 30°C and then rapidly frozen in liquid nitrogen before final storage at -20°C. Frozen sections were fixed and then incubated with a pepsin digest of either rabbit anti-(human α_2 HS glycoprotein) immunoglobulin G or a rabbit non-immune globulin as control, and then with the fluorescein-labelled Fab' fragment of a pig antibody to rabbit immunoglobulin G Fab'.

Some sections were fixed and stained by von Kossa's method for calcium, being counterstained with haematoxylin and chromotrope. Staining for calcium revealed a broad band of calcification in the periosteum adjacent to the hypertrophic cartilage of the shaft (Fig. 1*a*). There was also extensive calcification within the shaft and early signs of calcification in the hypertrophic cartilage. Wherever extracellular calcium was detected we observed very intense green fluorescent staining in sections which had been initially treated with the digest of the immune globulin (Fig. 1*b* and *c*). The intensity of this immunohistochemical staining was greatest where staining with von Kossa's reagent was most concentrated. Sections which had been treated with the control non-immune immunoglobulin digest showed almost no staining associated with extracellular

Table 1 Haemagglutination titres of various antisera against three plasma α globulins

Protein	Plasma concentration	Bone protein-coated cells	Reciprocal haemagglutination titre	
			Serum protein-coated cells	Control
α_2 macroglobulin	180 mg dl ⁻¹	≤ 4	2048	1024
α_1 antitrypsin	150 mg dl ⁻¹	≤ 4	512	512
α_2 HS glycoprotein	60 mg dl ⁻¹	512	128	≤ 4

*Average concentrations in adult human plasma of three α globulins. Antisera against those proteins were incubated with tanned sheep erythrocytes coated with human bone proteins; only antiserum against α_2 HS glycoprotein caused haemagglutination. If these antisera were first absorbed with protein this inhibited the capacity of the antiserum against α_2 HS glycoprotein to haemagglutinate tanned sheep erythrocytes coated with either bone protein (not shown) or serum protein compared with the non-adsorbed antiserum control.

sites, including those where calcification had taken or was taking place (Fig. 1d). These control specimens did exhibit a very low level of non-specific green fluorescent cellular staining of the kind also seen in the test sections.

These results show therefore that a plasma protein, α_2 HS glycoprotein, is a constituent of both foetal and adult human bone matrix and is incorporated and concentrated into bone presumably through interactions with one of the other components of the mineralising matrix. The function of this protein in bone tissue has not yet been established but it has been observed⁷ that human plasma α_2 HS glycoprotein possesses opsonic properties, as demonstrated by its capacity to promote increased phagocytosis of *Staphylococcus aureus* and *Escherichia coli* by human neutrophils *in vitro*. This would account for our own observations that plasma levels of α_2 HS glycoprotein, measured by radial immunodiffusion, fall markedly after various surgical procedures (I. R. D., unpublished); in the case of surgical damage to bone, plasma levels of this glycoprotein, corrected for plasma volume changes, fall to as little as half their pre-operative values. There is no evidence that this protein is present in areas where pre-osseous cartilage is degraded so that, if the opsonic property is utilised, it will most likely be in mediating the eventual resorption of the bone.

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Propagation of human wart virus in tissue culture

HUMAN wart (papilloma) virus (HWV) is an authentic human tumour virus. Based on morphology, it has been classified as a member of the papova group¹ of animal tumour viruses. Using electron microscopy, HWV has been found to be associated with human skin warts², laryngeal and venereal papillomas^{3,4} and epidermodysplasia verruciformis⁵. Moreover, experiments done with human volunteers at the beginning of this century^{6–8}, showed that filtered extracts of warts, laryngeal and venereal papillomas can cause skin warts in man. Since these early experiments, there has been little increase in our knowledge of HWV as a causative agent of either benign tumours or those which can progress to malignancy^{9,10}, mainly because of the lack of a system in which to propagate HWV *in vitro*¹⁰. This report describes the propagation of HWV in a tissue culture system.

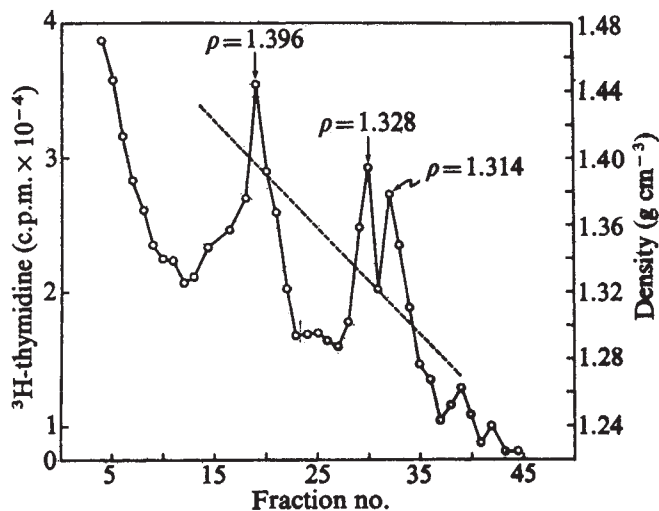


Fig. 1 Isopycnic centrifugation of HWV grown in tissue culture. BE cells were infected with HWV that has been passaged in tissue culture 11 times. ³H-thymidine (2 μ Ci ml⁻¹) was added to the culture fluid 2 h after infection. Rounded, detached cells along with culture medium were collected 5 d later; cells were disrupted by repeated freeze-thawing, clarified at 800 r.p.m., and virus was concentrated by centrifugation (SW27; 2 h, 25,000 r.p.m.). Resuspended viral pellet was centrifuged in CsCl (ρ 1.32 g cm⁻³) in an SW50 rotor for 72 h at 40,000 r.p.m.; fractions were collected from the bottom of the tube. Gradient density was determined by measuring refractive index of various fractions.

HWV is epitheliotropic. It infects epidermal cells, a wart probably being a clone of transformed descendants¹¹ of a single infected cell. Virus progeny are produced in epithelial cells *in vivo*². Our first choice was, therefore, to select a human epithelioid cell strain in which to attempt virus propagation *in vitro*. We have used a cell strain established by Dr M. Bean (Bean epithelial, BE) which, to our knowledge, is unique, as no other epithelioid cell strains have been established from human skin.

Virus was obtained from surgically removed samples of human skin warts, collected in tissue culture medium, incubated at 37 °C for 27 h, and either stored at –80 °C or used immediately. Pooled samples of wart tissue were minced and homogenised with carborundum with a mortar and pestle. Suspensions (10% w/v) were prepared according to a procedure described by Pass and Marcus¹².

To infect BE cells successfully *in vitro*, it was necessary to use cells in the logarithmic phase of growth and to allow the virus to absorb to the cells at low pH (6.5), and then to stress the cells by raising the pH rapidly to an alkaline level (8.0), after which the pH was allowed to fall slowly in a CO₂ incubator to pH 7.0–7.2. Alkaline pH stress was repeated 24 h after infection, and the HWV was thus successfully passaged 14 times in series. Unless cells were treated each time with these three successive pH stresses, serial passage of HWV with cytopathic effect (CPE) failed. The following changes were considered to constitute CPE. At 4–5 d after infection many rounded cells appeared which, in stained preparations, were seen to have intranuclear inclusions accompanied by nuclear vacuolisation. Infected cultures at this time also contained many clear areas surrounded by rounded cells that could be shown by immunofluorescence to contain viral antigens. Rounded cells eventually became detached and died; however, even with continued incubation for 3–4 weeks, only about 50% of the adjacent flattened cells showed similar changes. When the rounded cells were shaken off and examined by indirect immunofluorescence, using hyperimmune rabbit antisera prepared against virus purified from human warts, 90% showed positive nuclear fluorescence. When the remaining attached cells were removed with trypsin and similarly examined, 2–4% were positive. Mock-infected controls treated

in parallel with infected cultures showed neither cytopathic changes nor positive fluorescence.

As progression of the cytopathic changes to complete destruction of all cells in the culture was not observed, a titration method depending on a 50% dilution endpoint could not be relied on. In neutralisation tests carried out with undiluted tissue culture virus, however, cytopathic changes were never found when the tissue culture virus was pretreated with hyperimmune rabbit serum prepared against wart virus isolated from human tissues, or wart virus grown in tissue culture, and some human sera from patients with warts. The cytopathic changes were, however, always observed when tissue culture

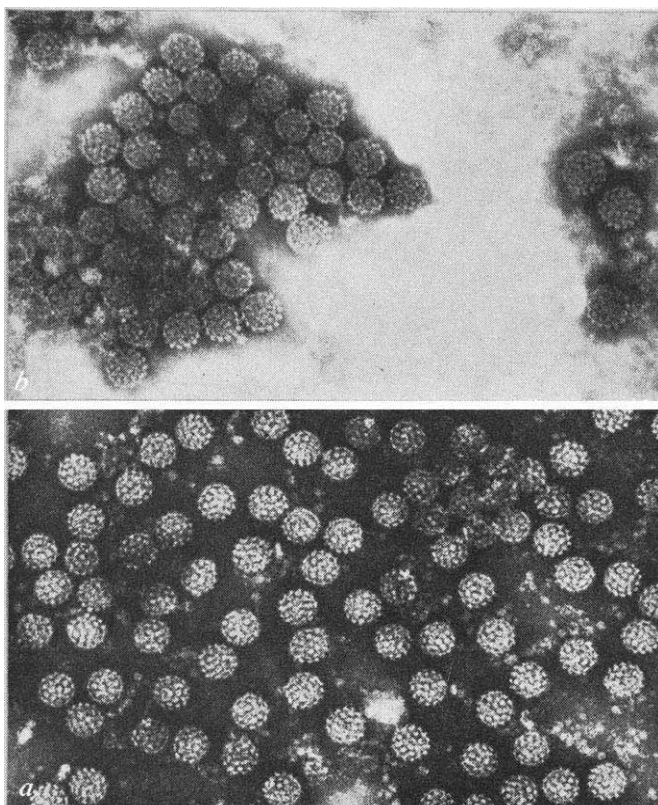


Fig. 2 Electron micrographs of HWV stained with sodium phosphotungstate, pH 7.0. *a*, Tissue culture virus from passage 7 (10^7 particles ml^{-1} concentrated from 400 ml supernatant). $\times 102,600$. *b*, Virus extracted from human warts (10^{13} virus particles ml^{-1}). $\times 102,600$. Both represent virions that were banded in CsCl gradient at a density of 1.33 g cm^{-3} . BE tissue culture cells were always infected as a suspension, originally with 10% (w/v) extract of pooled human skin warts (5×10^2 cells were infected with 0.5 ml virus suspension containing 5×10^{12} virus particles). After 2 h incubation and pH stress, the cells were plated in a 60 ml Petri dish and 4.5 ml of medium added. As cytopathic changes occurred at first rather rapidly, the cells and supernatant were collected after only 5 d. Cells suspended in the supernatant were freeze-thawed three times and then pelleted at 800 r.p.m. for 10 min. The pellet was hand-homogenised in 1 ml original supernatant and the whole sample was centrifuged at 800 r.p.m. for 10 min to remove debris. Final supernatant was then considered as virus stock at passage 0. (The original inoculum was therefore diluted 1:10 and if no virus was lost, its concentration could be $5 \times 10^{11} \text{ ml}^{-1}$.) Fresh cells were treated in the same way as before (but 2 ml of virus stock used for 1×10^6 cells) and the cells plated into 20 ounce plastic bottles diluting the cells in 20 ml medium. Many rounded cells appeared first after 5 d, when the medium was withdrawn and replaced with 20 ml fresh medium. Cultures were then incubated until more pronounced cytopathic changes were observed (up to 16 d) when they were processed as described for passage 0. The supernatant removed after 5 d was not used for further passage in this set of experiments. The procedure was then repeated at each virus passage. (Each virus passage therefore involves a 1:200 dilution factor. Thus, the original inoculum can be expected to be diluted out by passage 5–6.)

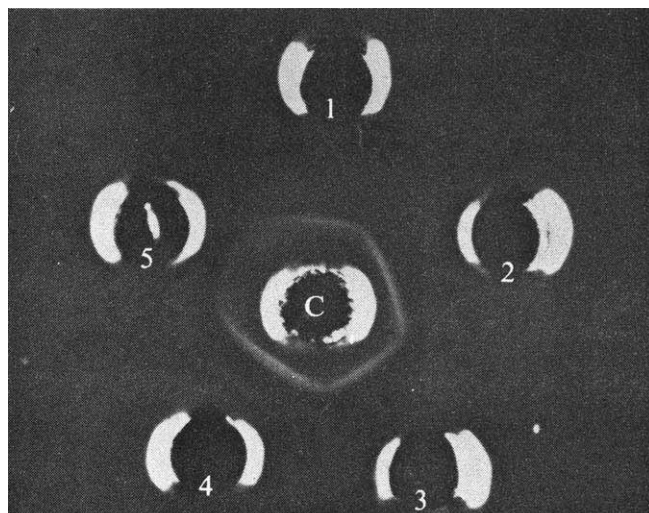


Fig. 3 Double immunodiffusion in 0.7% agarose gel. The centre well (C) contains purified HWV isolated from human wart tissue. The peripheral wells 1, 2, 3 and 4 contain rabbit antisera after two, three and four immunisations, respectively, with purified HWV isolated from the wart tissue. Well 5 was filled with rabbit antiserum after three immunisations with purified HWV grown in tissue culture. Purified viral pellet was resuspended in a small volume of Tris-EDTA buffer, pH 7.4, and sonicated for 60 s with a Biosonic sonicator. This was used as antigen for immunodiffusion studies. Rabbits were immunised with purified virus, emulsified in an equal volume of complete Freund's adjuvant and given subcutaneously in multiple sites. Four weeks later, the same amount of antigen in incomplete Freund's adjuvant was similarly injected. Subsequent immunisations were started 3 weeks later and given intravenously at 10 d intervals.

virus was similarly treated with foetal calf serum, preimmune rabbit sera, or sera from healthy blood donors.

To show that the virus was actively multiplying in culture and was not merely being carried along passively from culture to culture, we attempted to demonstrate incorporation of ^3H -thymidine into newly formed virions (Fig. 1).

Density gradient centrifugation of labelled virions in a CsCl gradient resulted in a split peak of radioactivity at buoyant density approximately 1.31 and 1.33 g cm^{-3} as well as another peak at a density of 1.39 g cm^{-3} . The 1.31 – 1.33 peak contained typical wart virus particles (Fig. 2*a*), whereas no virus was found in the peak corresponding to $\rho = 1.39 \text{ g cm}^{-3}$. Interestingly, the later peak contained mainly hexagonal structures with a dense interior, suggesting that they may represent viral cores. Heavier material sedimenting to the bottom of the tube remains to be investigated.

Gradient centrifugation of unlabelled virions, derived from tissue culture as well as from human wart extracts, usually showed three visible bands, two at the above described densities and a third at a density of approximately 1.29 g cm^{-3} which contains mainly empty particles. These findings are consistent with the observations of Pass and Maizel¹³ who found three visible viral bands at similar densities in virus preparations made from human plantar warts.

The tissue culture grown virus was identified as HWV by morphological and immunological analyses. The particles shown in Fig. 2*a* are identical in size and morphology to those of the viruses isolated from human warts (Fig. 2*b*). The average diameter of these two groups of particles is $532 \text{ \AA}$, a value which is larger than the size ($430 \text{ \AA}$) of the polyoma SV40 group of papovaviruses. Thus, by virtue of its larger size^{14,15}, the particles observed in this study may be distinguished from SV40 viruses.

Antisera made against the virus isolated from human warts, as well as antisera made against the virus propagated in tissue culture, gave a line of identity in immunodiffusion when reacted against purified virus from human warts (Fig. 3). Similar results were obtained with virus grown in tissue culture.

A second line of precipitation was occasionally seen in these immunodiffusion tests. None of these sera, however, reacted with SV40 viral antigens. Lack of cross-reactivity was also seen by immunofluorescence when SV40 infected cells were tested against anti-HWV serum and vice versa.

These observations strongly suggest that HWV has been propagated in tissue culture. This study may provide means for: further studies on the identity of viruses causing human warts, venereal warts and laryngeal papillomas^{16,17}; and comparing the properties of HWV with those of other human papovavirus isolates¹⁸⁻²⁰. Moreover, as warts are common complications in patients with primary and secondary immunodeficiencies and in patients under immunosuppression²¹, we believe that a system for the study of HWV *in vitro* may provide tools for the study of immune mechanisms responsible for the defence against virally-induced tumour in man.

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Persistence of viral DNA in human cell cultures infected with human papilloma virus

THE human papilloma (wart) virus produces a transmissible, benign neoplastic disease which is self-limiting and often regresses. Although rare, papillomas containing papovavirus-like particles may undergo malignant transformation. Papillomas in epidermodysplasia verruciformis and condyloma acuminatum can become neoplastic¹⁻⁴. Reports of the wart virus replicating in or transforming cells in culture, however, have not been substantiated⁵⁻⁷. Butel was unable to demonstrate cytopathic effect, the production of viral antigens or virus replication using a variety of cell lines and culture techniques⁸. Mild stimulation of cellular DNA synthesis, however, was observed in contact-inhibited embryonic human kidney cells after infection⁸. We also have been able to induce cellular DNA synthesis in confluent monolayers of a cell line derived from foetal human foreskin (NHP cells) following infection by wart virus.

Induction of DNA synthesis by papovaviruses may be essential for integration of viral DNA⁹⁻¹² and thus it is possible that during induction of cellular DNA synthesis by the wart virus, viral DNA was integrated into host DNA. Such genetic

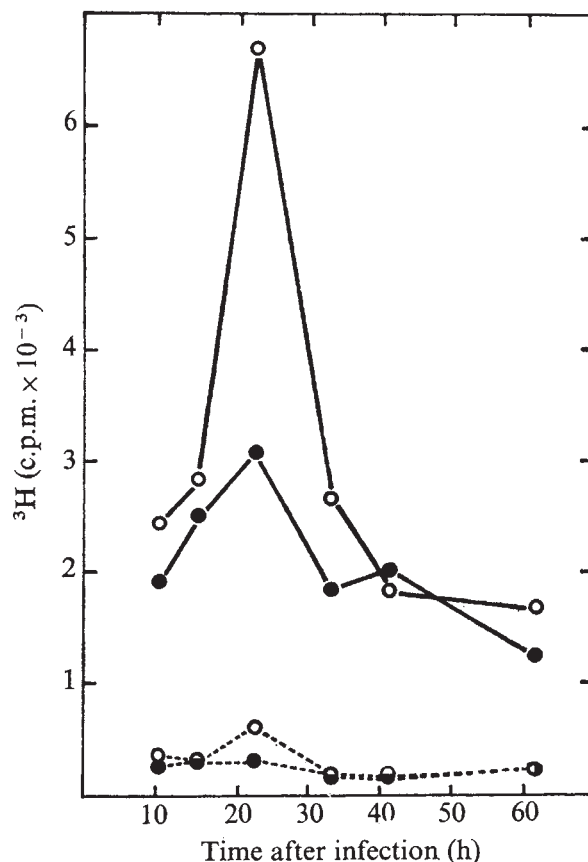


Fig. 1 Induction of cellular DNA synthesis by the human wart virus. Monolayers of NHP cells were grown in 15 × 60 mm dishes in Eagle's medium containing 10% foetal calf serum at 36 °C. When cell cultures were confluent, the temperature was decreased to 33 °C for 12 h. Duplicate cultures were washed with medium and either infected with 0.2 ml virus or overlaid with 0.2 ml medium for 1.5 h at 33 °C. Cultures were then overlaid with 5 ml medium containing 2% foetal calf serum and maintained at 33 °C. At various times after infection, cultures were labelled for 3 h with 1.0 ml Eagle's medium containing 5% dialysed horse serum and 10 μCi ml⁻¹ ³H-thymidine (6.7 Ci mmol⁻¹, New England Nuclear) followed by a 1 h chase with Eagle's medium containing 10% calf serum. Plates were washed twice with cold phosphate-buffered saline¹⁵, cells lysed and fractions separated according to the procedure of Hirt¹⁶. Hirt pellets and a portion of the Hirt supernatant fluid were made 1 N with 10 N NaOH and incubated for 60 min at 50 °C. Triplicate samples (0.025 ml) were spotted on filter paper disks (Schleicher and Schuell, 740E) and treated for TCA-precipitable materials using a batch technique¹⁷. Filter paper disks were counted in toluene scintillation fluid. ○—○, Infected pellet; ○---○, infected supernatant; ●—●, control pellet; ●---●, control supernatant.

information was detected using wart viral DNA labelled *in vitro* as a probe in DNA-DNA hybridisation studies. We found wart viral DNA persisting in cells 11 months after infection.

Virus was isolated from 5 g tissue homogenised in a Virtis at 45,000 r.p.m. at 4 °C for 30 min in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl followed by a second homogenisation (10,000 r.p.m., 4 °C, 5 min) in an equal volume of 1,1,2-trichlorotrifluoroethane¹³ (freon). The aqueous phase containing virus was separated from the freon phase and virus pelleted at 54,000g for 4 h at 4 °C. Pellets were suspended in CsCl (ρ = 1.33 g ml⁻¹ in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA) and centrifuged to equilibrium at 35,000 r.p.m. at 25 °C in the SW50 rotor. DNA was isolated following rupture of virions with 1% sodium dodecyl sulphate (50 °C, 15 min) and by extraction with phenol. DNA was precipitated with ethanol and supercoiled molecules isolated by CsCl-EtBr equilibrium centrifugation¹⁴.

Induction of cellular DNA synthesis by the wart virus is shown in Fig. 1. Twenty-four hours after infection there was a

Table 1 Summary of the reassociation of ^{32}P -labelled wart viral DNA in the presence of unlabelled cellular DNA

Experiment	DNA source	Cellular DNA (mg ml ⁻¹)	^{32}P -wart DNA (ng ml ⁻¹)	Factor of increased rate*	No. of unlabelled viral genome equivalents per diploid cell†
1	NHP	1.6	0.8	—	—
	<i>B. subtilis</i>	1.6	0.8	—	—
	Infected NHP	1.6	0.8	1.4	0.16
	<i>B. subtilis</i> + 2.0 genome equivalent unlabelled wart viral DNA per diploid cell	1.6	0.8	6.7	2.28
2	NHP	2.4	0.6	—	—
	Wi38	2.4	0.6	—	—
	Salmon sperm	2.4	0.6	—	—
	Infected NHP	2.4	0.6	2.0	0.20
	Infected Wi38	2.4	0.6	0.0	0.0
	Salmon sperm + 0.6 genome equivalent unlabelled wart viral DNA per diploid cell	2.4	0.6	3.8	0.56

*Increased rate relative to reassociation of ^{32}P -viral DNA in presence of control cellular DNA.†Calculated by the method of Gelb *et al.*²⁵.

t wofold increase in the amount of ^3H -thymidine incorporated into Hirt pellets of infected cultures compared with mock-infected controls, followed by a decrease to control levels by 32.5 h. Similar stimulation was observed in Hirt supernatant fluids. Examination of infected supernatant fluids in CsCl-EtBr equilibrium gradients and by CsCl velocity centrifugation, however, revealed neither superhelical DNA nor DNA with sedimentation rates expected for wart viral DNA.

DNA-DNA reassociation was used to show the presence of viral DNA sequences in infected cells which had been maintained in culture for several months. Cellular DNA was partially purified by the method of Meinke *et al.*¹⁷ and was subsequently treated with RNase (13 $\mu\text{g ml}^{-1}$, 37 °C, 30 min), then Pronase (25 $\mu\text{g ml}^{-1}$, 37 °C, 30 min) and phenol extracted. Cellular DNA was then sheared to an average single-stranded piece size of 560 nucleotides¹⁸.

To label wart viral DNA *in vitro*, DNA was first nicked with pancreatic DNase I at a ratio of 1,000:1 (w/w) in 50 mM

Tris-HCl, pH 7.4, 50 mM KCl, 5 mM MgCl_2 for 20 min at 37 °C. DNase was inactivated by heating solutions to 70 °C for 10 min. Nicked DNA was labelled in conditions of repair synthesis with *Escherichia coli* DNA polymerase I (refs 19 and 20). Specific activities of ^{32}P -TTP and ^{32}P -TTP + ^{32}P -dATP-labelled DNAs were 1.95×10^7 and 4.85×10^7 c.p.m. per μg DNA, respectively. Labelled DNAs had sedimentation rates in alkaline sucrose gradients of 5.9 and 6.3S. Sedimentation rates did not change during experiments. Reannealing of denatured labelled DNAs were at rates expected for viral DNAs with piece sizes as described²¹. ^{32}P -viral DNA reassociated to 90% in the presence of fivefold excess of nicked unlabelled wart DNA. SV40 and wart DNA have the same guanine+cytosine content (41%)^{22,23} and *in vitro* labelled wart DNA had a similar T_m (86.0 °C), as determined by hydroxyapatite chromatography¹⁸, as did *in vivo* labelled SV40 DNA (86.6 °C).

The kinetics of DNA-DNA reassociation follows the

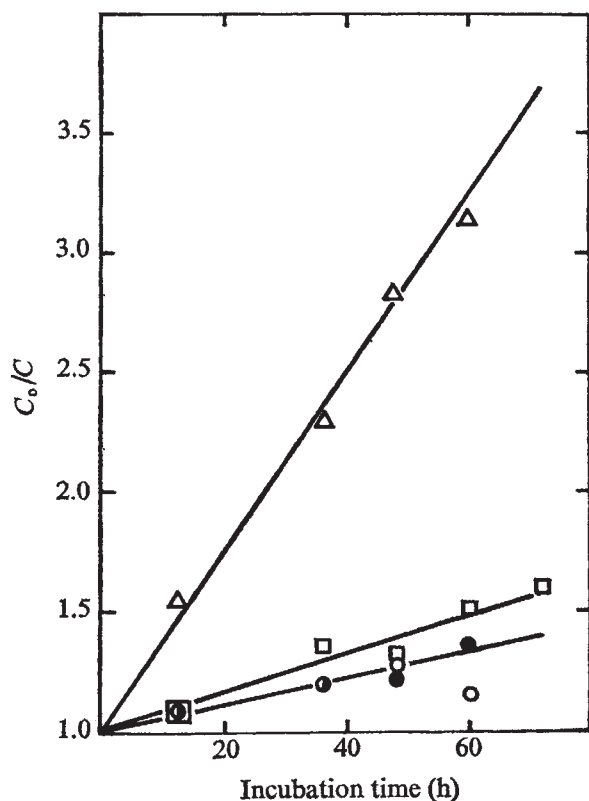


Fig. 2 Reassociation of ^{32}P -labelled wart DNA in the presence of unlabelled DNA from infected NHP cells, mock-infected NHP cells or *B. subtilis*. Nicked viral DNA (1 μg) was suspended in a volume of 1.0 ml containing 66 μmol potassium phosphate, pH 7.4, 6.6 μmol MgCl_2 , 1 μmol β -mercaptoethanol, 0.05 μmol each of dATP, dGTP, dCTP, and 2 mCi ^{32}P -TTP (114.8 Ci mmol⁻¹) or 0.05 μmol each of dGTP and dCTP with 2 mCi each ^{32}P -TTP and ^{32}P -dATP (118.0 Ci mmol⁻¹, New England Nuclear). DNA repair synthesis was initiated by addition of 20 units *E. coli* DNA polymerase I (Grand Island Biological) for 75 min at 15 °C and terminated with 1% Sarkosyl. Labelled DNA was separated from low molecular weight material by chromatography on Sephadex G-50 equilibrated with 0.1% Sarkosyl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA and deproteinized by phenol extraction. Solutions of cellular DNA (1.6 mg ml⁻¹) and ^{32}P -labelled viral DNA (0.8 ng ml⁻¹) were denatured in 0.48 M sodium phosphate, pH 6.8, 0.05% SDS, 0.001 M EDTA at 109 °C for 5 min in screw-capped tubes. Solutions of single-stranded DNA were then allowed to reassociate at 64 °C. At various times samples were removed and diluted to 2.0 ml in 0.14 M sodium phosphate, pH 6.8, 0.05% SDS. Single-stranded DNA was separated from reassociated double-stranded DNA by hydroxyapatite column chromatography (10 $\times$ 15 mm) at 60 °C²⁴. Single-stranded DNA was eluted from the column with five 2.0 ml washes of 0.14 M sodium phosphate, pH 6.8, 0.05% SDS; reassociated double-stranded DNA eluted with four 2.0 ml washes of 0.48 M sodium phosphate, pH 6.8, 0.05% SDS. Radioactivity was determined for each wash by addition of 15 ml toluene-Triton X-100 (2:1 v/v) scintillation fluid containing Cab-O-Sil (Packard). $\circ$, Mock-infected NHP cellular DNA; $\square$, infected NHP cellular DNA; $\bullet$, *B. subtilis* DNA; $\triangle$, *B. subtilis* DNA with 4.0 ng ml⁻¹ unlabelled nicked wart viral DNA. Each point represents about 500 c.p.m.

equation $C/C_0 = 1/(1 + KC_0t)^4$ (ref. 24); C_0 is the total ^{32}P -viral DNA concentration, C is the concentration of single-stranded ^{32}P -viral DNA, t is time, and K is the reassociation constant. A plot of C_0/C against t results in a straight line with the slope proportional to C_0 . The rates of reassociation of ^{32}P -labelled wart DNA in the presence of either *Bacillus subtilis* DNA or mock-infected NHP cellular DNA were very similar, indicating that there is no homology between NHP cellular DNA and wart viral DNA (Fig. 2). Addition of 4.0 ng ml^{-1} unlabelled nicked wart DNA (2.0 copies viral DNA per diploid cell) to a solution of ^{32}P -labelled wart DNA (0.8 ng ml^{-1}) and *B. subtilis* DNA increased the rate of reassociation of ^{32}P -labelled wart DNA by a factor of 6.7, which is in agreement with the expected value of 6.0. When denatured ^{32}P -labelled viral DNA was allowed to reassociate in the presence of DNA derived from infected NHP cells, the rate of reassociation was 1.4 times greater than controls. In another experiment, control cellular DNAs consisted of salmon sperm, human lung (Wi38) and NHP. The rate of reassociation of ^{32}P -labelled wart DNA was similar in each instance. The rate of reannealing of ^{32}P -labelled viral DNA in the presence of infected NHP cellular DNA, however, was twofold greater than controls. Interestingly, no wart viral DNA sequences could be detected in Wi38 cells infected in the same manner as NHP cells. The results are summarised in Table 1. The increases in the reassociation rates correspond to 0.18 wart viral genome equivalents per infected NHP cell.

The increase in the reassociation rate could not be the result of the presence of virus inoculum as input virus would have been diluted beyond detection by cell replication by the methods used. Cells used in these experiments had undergone 15 to 60 doublings after infection. The maximum possible multiplicity of infection as determined by particle counts was about 100 virions per cell. After 15 doublings, there would only be 0.003 viral genome equivalents per cell, which is well below the values detected. The stable persistence of viral genetic information in infected cells must, therefore, be the result of replication of viral DNA sequences which may be integrated into the host genome.

As infected NHP cells were not cloned, detection of 0.18 wart viral genome equivalents per diploid cell indicates that each cell contains a portion of the viral genome or that only some cells carry a complete genome. It is possible that cells carrying the viral DNA have no selective advantage over that of uninfected cells in the culture and thus remain in relatively low numbers.

SV40 DNA can persist in abortively transformed mouse cells²⁶ and recently, it was shown that SV40 DNA injected into the blastocoel of mouse blastocysts resulted in persistence of SV40 DNA sequences through subsequent maturation to adult mice²⁰. Our results indicate a similar situation may exist for the human wart virus in that there is a persistence of wart gene sequences in infected NHP cells.

As the human wart virus is the only known tumour agent of man, it is of interest that wart viral DNA can persist in a cryptic state in infected NHP cells *in vitro*. Although cells seem to be normal with respect to growth and morphology the possibility exists that under proper conditions the latent viral DNA sequences could become 'derepressed' and induce cell growth characteristic of transformed cells. These results would indicate that the ubiquitous human wart virus should be given serious consideration as a possible agent of neoplastic disease in man.

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Effect of bromodeoxyuridine on redundancy of ribosomal RNA cistrons of *Drosophila virilis*

THERE have been numerous demonstrations that the thymidine analogue, 5-bromodeoxyuridine (BrdU), can affect the progress of cellular events in eukaryotes. It has been suggested that the incorporation of the base analogue changes the secondary structure of DNA and/or chromatin recognition sites¹⁻³. Support for this view has come from studies which have shown a BrdU substitution effect on the physical properties of isolated chromatin^{4,5} and alterations in the patterns of RNA transcription⁶⁻⁹. In some instances, a correlation between the onset of DNA synthesis and the effect of BrdU has been observed^{1,3}. In addition, several studies have shown that the drug can alter the process of DNA replication^{8,10,11}. Thus, it has been argued that changes in the replication of DNA may precede or lead directly to the expression of BrdU sensitivity.

We have initiated a study of the influence of BrdU on the redundancy of the ribosomal RNA (rRNA) cistrons of *Drosophila*. The rDNAs have been localised to the nucleolus organiser regions of the *Drosophila* karyotype¹², and can be manipulated genetically. The redundancy of the rRNA cistrons can be measured by hybridisation techniques and evidence of mechanisms which control both the replication¹³ and transcription^{14,15} of these sequences has been presented. Thus, the interaction of transcriptional and replicational influences of BrdU can be examined with regard to the behaviour of a single DNA sequence. In this report we describe an effect of BrdU on the redundancy of rRNA cistrons of *D. virilis*. *D. virilis* was chosen because a substantial portion of the diploid genome contains A-T-rich redundant sequences. These sequences have been localised to the heterochromatic regions of the *D. virilis* karyotype¹⁶ which also contain the rRNA cistrons. It was argued that A-T-rich regions may preferentially incorporate BrdU.

BrdU was added to the medium of *D. virilis* larvae on days 5 and 7 after the parental adults had been placed on the food. The redundancy of the rRNA cistrons in DNA from the BrdU-treated larval material did not differ significantly from that of the untreated larval control populations (Table 1, experiment 1). When drug-treated larvae from this experimental population were reared to the adult stage, however, a substantially smaller fraction of the DNA hybridised with rRNA compared with controls. Note that the rRNA cistron redundancy is repeatedly

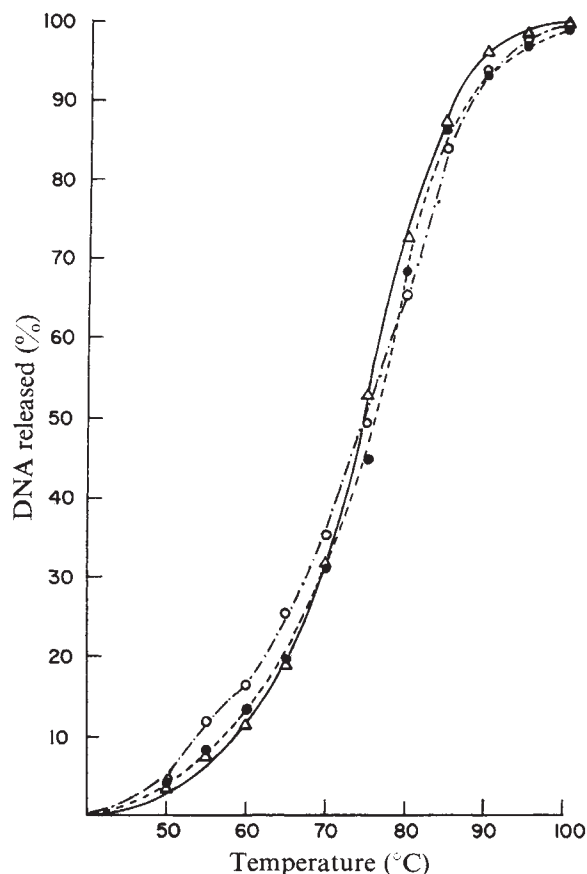


Fig. 1 Thermal stability of ^3H -rRNA-DNA hybrids. Denatured DNA from *D. melanogaster*, *D. virilis*, and BrdU-treated *D. virilis* (days 5 and 7, experiment 1, Table 1) adults was loaded on to filters and the hybridisation reactions performed as described in the legend to Table 1. After RNase treatments the filters were washed and placed in glass vials with 3 ml $1 \times \text{SSC}$ and incubated in a water bath. Starting at 45°C , the temperature was raised in 5° increments and maintained for 5 min before removing the solution and replacing it with fresh SSC at the same temperature. Counts released from the filters were determined using Aquasol (New England Nuclear). *D. melanogaster* ^3H -rRNA hybrids formed with *D. melanogaster* DNA (Δ); *D. virilis* adult DNA ($\bullet$); BrdU-treated (days 5 and 7) *D. virilis* adults ($\circ$).

found to be lower in DNA from larval tissues than DNA from adult tissues of *D. virilis*. Similar results have been reported for other species of *Drosophila*²¹. The observation was anticipated because the rDNAs are underreplicated in polytene cells^{7,13}, and these cells are most prominent in the larval stages.

When the experiments were repeated, two populations were established for drug treatment. In one case, drug was spread on the food on days 5 and 7 after the parental adults began egg laying, as in the previous experiment. Drug was administered to the second group on days 2 and 5, after egg laying had indicated whether the BrdU effect was limited to the postlarval stages. The results obtained from treatments on days 5 and 7 (Table 1, experiment 2) agree with those of experiment 1. There was no substantial difference in rDNA content between larval BrdU-treated and control DNAs, but a significant decrease in the rDNAs from treatments on days 5 and 7 (Table 1, experiment 2) difference in rDNA content between larval BrdU-treated treatments 1 and 2 probably reflects variations in the number of larvae grown on the food and the time over which the drug was available to the larvae. DNA extracted from larvae treated with BrdU on days 2 and 5 (Table 1) contained significantly lower rDNA redundancy (18%) than control larvae. DNA from the adults of this population, however, showed about one-half the effect ($\sim 9\%$) compared with DNA from untreated adults. Thus, BrdU influences the redundancy of the rRNA cistrons in both

adult and larval tissues. In the conditions used, however, the effect, if prominent in larval material, was not entirely sustained into the adult tissues.

Note that we have made no effort to screen for any of the development aberrations reported by other workers using BrdU on developing *Drosophila* systems^{17,18}. BrdU-treated adults are not phenotypically bobbed. The major gross effect of our BrdU treatments on larvae is an increase in the time required to reach adult eclosure. Viability, fertility, and sex ratios of eclosing BrdU-treated adults are not significantly affected by these experimental conditions (D. Durica and H.M.K., unpublished).

Two controls were performed with regard to the artefacts which BrdU might cause in the measurement of hybrids in our heterologous DNA-RNA reactions. In the first, the thermal stability of the hybrids formed between *D. melanogaster* ^3H -rRNA and DNA from BrdU-treated and control *D. virilis* adults was compared with homologous *D. melanogaster* hybrids. A typical melting profile from each type of hybrid is presented in Fig. 1, which shows that the thermal stability of the heterologous hybrid is comparable with the stability of homologous *D. melanogaster* DNA-RNA complexes. Furthermore, the DNA from BrdU-treated material formed hybrids of comparable quality to those formed by the other DNAs. These thermal stability studies, however, were performed on RNase-resistant hybrids. Thus, it might be argued that BrdU and control *D. virilis* DNAs have the same number of rRNA cistrons, but the hybrids formed by DNAs from BrdU-treated material are more sensitive to RNase. To examine this possibility, the radioactivity (^3H) associated with filters was determined before and after RNase treatment for DNAs from BrdU-treated adults (Table 1, experiment 2) and controls. There was no difference in RNase sensitivity of the DNA-RNA hybrids formed by these DNA samples. Thus, the hybrids formed by DNA from BrdU-treated material seem to be neither less stable nor more

Table 1 Hybridisation of *D. melanogaster* ^3H -rRNA to DNA of BrdU-treated *D. virilis*

	Day of BrdU addition	% DNA in hybrid		
		Experiment 1	Experiment 2	% Change
Larval	2 and 5	—	0.290	18.3
	5 and 7	0.356	0.351	0-1.1
	Control	0.355	0.355	—
Adult	2 and 5	—	0.352	9.5
	5 and 7	0.212	0.271	45.5-30.3
	Control	0.389	0.389	—

D. virilis were grown on standard corn meal, agar, and sucrose media at 22°C in half-pint bottles. Adults were placed on fresh media for 24 h, the bottles were then cleared, and at various times 2 ml 10^{-3} M BrdU solution (Sigma) were spread on top of the media. This concentration and means of application was chosen on the basis of other BrdU studies in developing *Drosophila* tissues^{17,18}, and some preliminary observation communicated by Juan Valencia (University of Wisconsin, Madison). Larvae in the third instar were collected by flotation away from the food with 20% sucrose and washed extensively in distilled water. DNA extractions from larvae and adults were performed as described elsewhere¹⁹. Nitrocellulose filters loaded with about 20 μg denatured DNA were incubated for 12 h at 67°C in $4 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% sodium lauryl sulphate, and 3.75 $\mu\text{g ml}^{-1}$ (68,800 c.p.m. μg^{-1}) ^3H -rRNA. ^3H -rRNA was prepared from *D. melanogaster* cell lines using the technique of Brown and Litt²⁰ (gift of Dr L. Weber, SUNY, Albany). The filters were washed with $2 \times \text{SSC}$, treated with 20 $\mu\text{g ml}^{-1}$ RNase A at 25°C for 2 h, washed again with $2 \times \text{SSC}$, and counted in Liquifluor (New England Nuclear) after drying. Scintillants were removed from the filters by several washings with toluene and ether, and the filter-bound DNA was determined using the procedure of Kissane and Robbins²⁶. All DNA preparations were tested in the same reaction vessel. A minimum of six measurements was made for each type of DNA. Untreated larval and adult DNA preparations were used as internal standards to compare hybridisations of experiments 1 and 2. Maximum s.e.; experiment 1, ± 0.022 ; experiment 2, ± 0.013 .

RNase-sensitive than the hybrids formed by DNA from untreated tissues. Therefore, the differences measured in the hybridisation reactions must reflect changes in the redundancy of the rRNA cistrons associated with the presence of BrdU in the developing larvae.

We may only speculate as to the mechanism(s) which produce this BrdU effect. The redundancy of the rRNA cistrons seems to be subject to control. The number of rounds of DNA synthesis which the rDNA sequences undergo differs significantly from other nucleotide sequences in polytene chromosomes^{7,13,22}. Further, the cells of *D. melanogaster* which bear a substantial deletion of the rDNAs when diploid, possess nearly wild-type levels of rRNA cistrons in polytene tissue¹³. In addition, alteration in rDNA redundancy of non-polytene cells can occur in *D. melanogaster*²³. It has been suggested that such rDNA magnifications occur through unequal exchange events between sister chromatids before formation of meiocytes²⁴. The presence of BrdU in developing larvae may affect any of the events normally associated with establishing and maintaining the redundancy of rRNA cistrons in polytene and diploid cells. To date we have no direct evidence that the BrdU effect described here is limited to particular tissues or cell types; nor is it clear that the effect is limited to rRNA cistrons. Preferential incorporation of BrdU into highly and intermediately repeated DNA sequences has been observed in tissue culture cells grown at low drug concentrations^{6,25}. Such preferential incorporation may explain the specificity of the action of BrdU in this case, and further suggest that the A-T-rich redundant sequences which surround the rDNA may also be influenced by the drug. Experiments designed to examine some of these possibilities are in progress.

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Inhibition of myogenesis in a rat myoblast line by 5-bromodeoxyuridine

5-BROMODEOXYURIDINE (BrdU) at very low concentrations inhibits the expression of cell or tissue-specific characteristics of several differentiating systems (for reviews see refs 1 and 2) including myoblasts³⁻⁶, and was postulated to cause its anti-differentiation effects by being incorporated into DNA segments²⁻⁶. Available data show, however, that in some cell types, the primary effects of BrdU may be mediated by means other than its incorporation with the genome^{7,8}. To determine whether template modification by BrdU *per se* is the cause of inhibition of myogenesis, we have investigated the reversal of inhibition by BrdU by various naturally occurring nucleosides, and show that deoxycytidine (and deoxyuridine) reverses the inhibition of myogenesis caused by BrdU without affecting its incorporation into cellular DNA. It seems likely, therefore, that template modification by BrdU may not be the sole cause of the inhibition of fusion of myoblasts brought about by this compound.

A clone, L6B, which was isolated from Yaffe's⁹ rat skeletal muscle myoblast cell line, L6, and which was stable with regard to its fusion properties, was used throughout. Concentrations of BrdU higher than 1 μ M during growth and division of the myoblasts completely abolish their differentiation into myotubes (Fig. 1). Below 1 μ M BrdU has very little effect on myogenesis. As with the chick myoblasts⁵ and other cell types¹⁰, L6B cells reach confluence in the presence of 3.5-16 μ M BrdU but are very much flattened. The doubling time and plating efficiency of L6B myoblasts remains unaltered at concentrations of BrdU which completely inhibit fusion (Fig. 1a). The inhibition of differentiation by BrdU is reversed

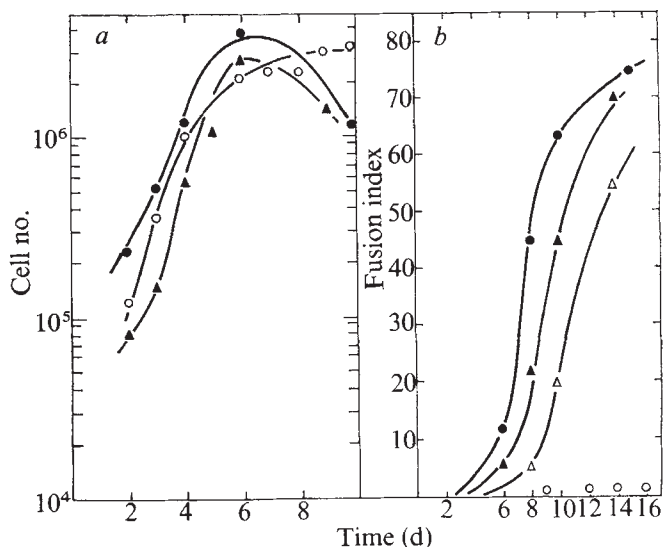


Fig. 1 Effect of BrdU and deoxycytidine on growth (a) and fusion (b) of myoblasts. *a*, About 10⁵ cells of clone L6B were grown in 250 ml Falcon culture flasks in 20 ml Dulbecco modified Eagle's (DME) medium supplemented with 10% horse serum, 50 μ g ml⁻¹ gentamycin and the nucleosides to be tested. The cells were incubated at 37 °C in an atmosphere of 10% CO₂ in air. The culture medium was replaced after the first day of plating and every 4th day thereafter. For growth measurement the cells were dissociated using 0.1% trypsin and counted in a haemocytometer. *b*, To obtain the fusion index cells were plated in 6 cm diameter plastic tissue culture dishes at a density of 3.5 $\times$ 10³ cells per cm² in 4 ml DME containing 10% horse serum and the required additives. At the indicated times, cells were washed with citrate saline, fixed with methanol, dried, and stained with Ehrlich's eosin-haematoxylin. Fusion index was determined according to the procedure of Morris and Cole¹⁷ by counting nuclei in single cells and myotubes in 32 fields (0.084 mm² per field) in early stages of fusion, and in 15-20 fields later. A cell was scored as a myotube if it contained three or more nuclei within the same cell. ●, No additions; ○, 3.2 μ M BrdU; ▲, 3.2 μ M BrdU plus 400 μ M deoxycytidine; △, 16 μ M BrdU plus 400 μ M deoxycytidine.

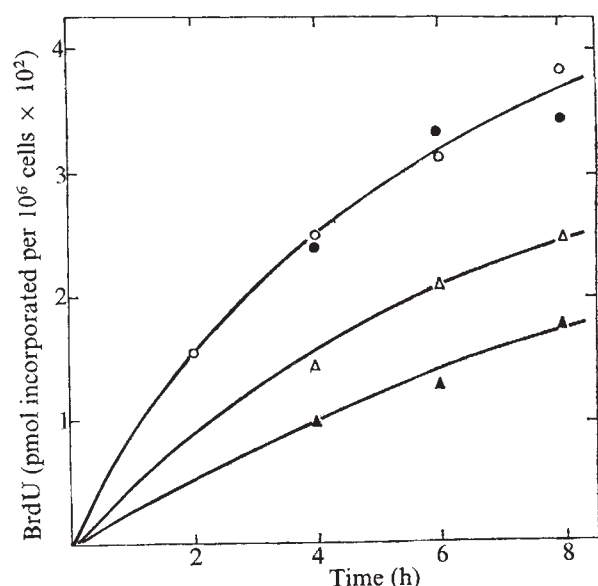


Fig. 2 Effect of nucleosides on the incorporation of BrdU in cells. L6B myoblasts (10^6 cells) were grown for 3 d in 35×10 mm, 6-well Linbro dishes in 5 ml DME containing 10% horse serum. Cells were washed consecutively with phosphate-buffered saline and citrate saline and then exposed to 3 ml growth medium containing $3.2 \mu\text{M}$ ($0.1 \mu\text{Ci}$) ^{14}C -BrdU ($50.6 \text{ mCi mmol}^{-1}$) and other nucleosides at 37°C . At the indicated intervals the cells were washed twice with saline and then treated for 15 min with 0.5 ml cold, 10% TCA. Cells were once again washed with saline to remove the TCA, dissolved in 0.5 ml 2 N NaOH, and counted. ○, BrdU alone; ●, BrdU plus 0.4 mM deoxycytidine; △, BrdU plus 1 μM thymidine; ▲, BrdU plus 2 μM thymidine.

to a large extent by the presence during growth of deoxycytidine, deoxyuridine or thymidine (Table 1).

Tested by themselves or together with BrdU at concentrations listed in Table 1, the nucleosides effective in restoring fusion do not cause any significant alterations in growth and plating efficiency of L6B myoblasts (Fig. 1a). Inhibition caused by higher concentrations of BrdU ($16 \mu\text{M}$) is also reversed by 400 μM deoxycytidine but the time required for maximal fusion is prolonged (Fig. 1b). Note that not only is the fusion potential of myoblasts restored in the presence of deoxycytidine, but the myotube-specific enzyme, creatine phosphokinase (CPK) also appears and increases in the fused myoblasts. As an example myoblasts in the presence of $3.2 \mu\text{M}$ BrdU show a specific activity (μmol product formed per min per mg protein) for CPK of less than 0.02, but in myotubes produced in the presence of BrdU and 400 μM deoxycytidine the specific activity rises to about 0.35.

The simplest way to explain the reversal by the nucleosides of BrdU-induced inhibition of differentiation is to assume that they inhibit the entry and subsequent incorporation with DNA of BrdU inside the cells. Steck *et al.*¹¹ have demonstrated that

Table 1 Effect of various nucleosides on myoblast differentiation in the presence of BrdU

Addition	Concentration (μM)	Fusion index
None	—	0.2
Deoxycytidine	4	21 ± 2
	40	46 ± 5
	400	46 ± 6
Deoxyuridine	33	31 ± 4
Thymidine	0.5	12 ± 2
	1	52 ± 5
Thymine	1	17 ± 3
Cytidine	400	19 ± 3
Uridine	400	1 ± 0.4
Guanine	400	5 ± 2
Adenine	400	4 ± 2

Cells of clone L6B were plated in the presence of $3.5 \mu\text{M}$ BrdU and the nucleoside to be tested. Fusion index was quantitated on day 10 after growth. Fusion index in the absence of BrdU was about 50. Details of experimental procedures are given in Fig. 1.

in various cultured cell lines uptake of any given nucleoside is inhibited by other heterologous nucleosides. In L6B myoblasts, however, only thymidine (but not thymine) inhibits the uptake of BrdU into the acid-insoluble material of the cell. We have ascertained that this material is probably DNA because treatment with DNase releases radioactivity in an acid-soluble form. Deoxycytidine (400 μM), however, has no effect on the uptake of BrdU (Fig. 2).

This suggests that thymidine and deoxycytidine reversal may occur through a pathway which is independent of the level of incorporation of BrdU in DNA. To investigate these possibilities further we carried out the following experiments. Myoblasts were grown for one to four generations (in different experiments) in the presence of $3.2 \mu\text{M}$ ^{14}C -BrdU, $3.2 \mu\text{M}$ ^{14}C -BrdU plus 1 μM cold thymidine, and $3.2 \mu\text{M}$ ^{14}C -BrdU plus 0.4 mM deoxycytidine. ^{14}C -BrdU ($50.6 \text{ mCi mmol}^{-1}$) was used at a level of 1 μCi in each case. At the end of the required time the cells (about 10^6) were collected using EDTA, and nuclei were isolated and lysed in 0.5% SDS as described previously¹². The DNA was banded by centrifugation in neutral CsCl density gradients and the specific activities (sum of c.p.m. in each DNA-containing fraction divided by the sum of A_{260} of the same fractions) computed¹². The DNA of control cultures (without BrdU or other additions) banded at a density of 1.703 g cm^{-3} and that of cells grown with BrdU ($3.2 \mu\text{M}$) for two generations at 1.708 g cm^{-3} . The amount of BrdU substitution of DNA was thus not more than 1–2% (ref. 13). The small amount of BrdU incorporated in our experimental conditions may be caused by the presence internally of sufficiently large pools of nucleotides in L6B myoblasts which interfere with the uptake or further metabolism of BrdU.

Whatever the reasons for the low incorporation of BrdU the significant finding was that the specific activity of DNA from cells grown in the presence of $3.2 \mu\text{M}$ BrdU in different experiments was approximately the same in myoblasts grown without (4.3×10^4), or with 0.4 mM deoxycytidine (4.7×10^4). We conclude that inhibition of fusion of myoblasts and reversal of this inhibition was not dependent on the substitution of thymidine residues in DNA by BrdU. A decrease in the level of BrdU incorporation in the presence of thymidine was found in our experiments (specific activity 3.2×10^4) and was expected in view of the BrdU uptake experiments presented earlier (Fig. 2). It is likely that differentiation of myoblasts is restored in the presence of thymidine (Table 1) either because inhibitory amounts of BrdU are not able to accumulate inside the cells (see later) or thymidine itself is able to counteract, like deoxycytidine, the inhibitory effect of BrdU on the yet unidentified events leading to fusion.

If it is agreed that BrdU substitution in DNA is probably not the cause of inhibition of myogenesis, as the results at least with deoxycytidine show, what are the conceivable mechanisms for BrdU inhibition of fusion? We propose that BrdU in a phosphorylated form at low concentrations inhibits some of the glycosyltransferases of the myoblasts which are responsible for the biosynthesis of fusion-specific glycoproteins or glycolipids and this inhibition is reversed by several pyrimidines related to the sugar donors (UDP-glucose, CMP-N-acetylneuraminic acid, and so on) of macromolecular synthesis. In support of this proposal it may be stated that several nucleoside analogues, such as puromycin¹⁴ and cytosine arabinoside¹⁵ have been shown to inhibit some glycosyltransferases in mammalian cells. In addition, it has been reported¹⁶ that chick embryo fibroblasts grown in the presence of BrdU become agglutinable by concanavalin A, which again suggests that alteration of sugar-containing macromolecules does occur in the presence of BrdU.

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Reduction in sympathetic nervous activity as a mechanism for hypotensive effect of propranolol

THE β -adrenoceptor antagonist propranolol is an effective anti-hypertensive drug in man¹ but it is not known how the fall in blood pressure is produced². There is a decrease in plasma renin activity³ and in cardiac output immediately on starting propranolol therapy but neither of these effects can explain fully the hypotensive action of the drug which is delayed in onset and associated with a reduction in peripheral vascular resistance⁴. It has been suggested that the fall in blood pressure after propranolol results from blockade of β -like adrenoceptors within the central nervous system⁵. Thus, intracerebroventricular injection of propranolol lowers blood pressure in conscious cats⁶ and rabbits⁶, an effect which is specific for the β -blocking (—) isomer of the drug⁷. The effective concentration of propranolol achieved in the hypothalamus by this route of administration is within the range predicted for hypertensive patients after chronic oral therapy⁸. We now present direct evidence that propranolol diminishes sympathetic nerve activity in the rabbit and that this central effect contributes to the hypotensive action of the drug.

Electrodes were implanted in the greater splanchnic nerve of rabbits weighing 3–4 kg (ref. 9). After a recovery period of at least 4 d a catheter was inserted under local anaesthesia into the central artery of an ear and used for measurement of blood pressure. Another catheter was inserted into the marginal vein of the other ear for administration of drugs. Each conscious unrestrained animal was placed in an individual cage and left undisturbed for 1 h. Integrated splanchnic nerve activity and arterial pressure were then recorded continuously for a 3-h period. The first hour served for control observations. After these, intravenous infusion of one of four solutions was begun and continued at a rate of 3 ml h⁻¹ for the remaining 2 h. The solutions used were saline, (±)-propranolol, (+)-propranolol and sodium nitroprusside, sufficient of each drug being dissolved in saline to enable an infusion rate of 1 mg kg⁻¹ h⁻¹. Each treatment was given to eight animals. Splanchnic nerve

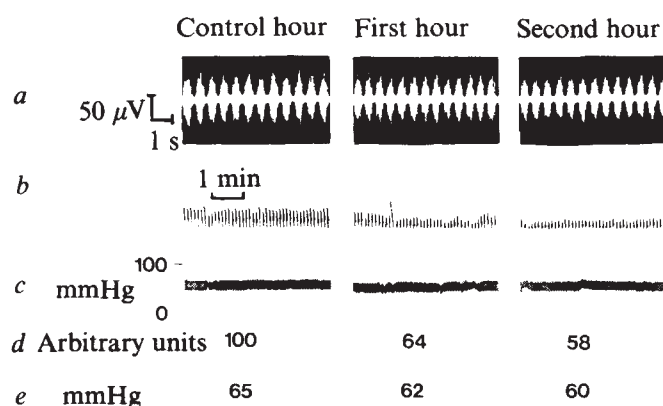


Fig. 1 Effect of (±)-propranolol infusion (1 mg kg⁻¹ h⁻¹ intravenously) on sympathetic nerve activity and arterial pressure in a conscious rabbit. *a*, Splanchnic nerve discharges as original oscilloscope records and as integrated activity over 6-s periods (*b*); *c*, arterial pressure (each record was taken at the end of the time period indicated); *d*, mean values of integrated splanchnic nerve activity per hour and of mean blood pressure measured over the complete hour by planimetry (*e*).

activity during each hour was summed by planimetry of the record, and the values during the infusion hours expressed as a percentage of that during the pretreatment hour. The systolic and diastolic arterial pressure traces were treated similarly except that the results were expressed in terms of mean arterial pressure, calculated as diastolic pressure plus one third of the pulse pressure.

Table 1 shows the effect of the four treatments on splanchnic nerve activity and mean arterial pressure. Neither saline nor (+)-propranolol, the non- β -blocking isomer of propranolol, altered these parameters significantly. Sodium nitroprusside, a vasodilator drug which relaxes vascular smooth muscle¹⁰, reduced arterial pressure. An increase in preganglionic sympathetic nerve activity accompanied the fall in blood pressure, indicating that the preparation is sensitive enough to detect reflex changes which are the expected response to a peripherally mediated hypotension.

By contrast, a different pattern of response was seen with (±)-propranolol which decreased both blood pressure and splanchnic nerve discharges. Figure 1 shows the original record of such an experiment, illustrating the parallel fall in preganglionic sympathetic nerve activity and blood pressure during (±)-propranolol infusion.

As sympathetic nerve activity was recorded from a preganglionic nerve, the site of the sympatho-inhibitory action of propranolol must be in the central nervous system. The only other possible explanation—that the drug increases the sensitivity of the arterial baroreceptors—seems very unlikely. Therefore, we suggest that blockade of central β -adrenoceptors brings about a reduction in sympathetic nervous tone and that this action could explain the antihypertensive effect of propranolol.

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Table 1 Effect of intravenous (+)- and (±)-propranolol, sodium nitroprusside and saline on mean arterial pressure (MAP) and splanchnic nerve activity (SNA) in conscious rabbits

Infusion	No. of animals	First hour		Second hour	
		MAP	SNA	MAP	SNA
Saline	8	97±2	111±5	99±3	104±16
(+)-Propranolol	8	101±2	122±17	99±4	132±24
(±)-Propranolol	8	94±3	68±11*	87±2†	52±9*
Sodium nitroprusside	8	74±1†	178±30*	73±2†	199±38*

Values are the mean ±s.e.m. of the average during one hour expressed as a percentage of the average during the hour pretreatment. Significant differences from saline control group: **P* < 0.05; †*P* < 0.01 (Student's unpaired *t* test).

reviews

Is research management really as boring as this book makes it sound? "Finally he should desirably exercise some flair which stamps his department with an identity, making it recognisable as a unit with a reputation for excellence", writes Mr White at the end of less than a page devoted to the attributes of a research leader. Flair is the ingredient conspicuously missing from what is really a rather expensive handbook of information for the research manager, long on lists of research laboratories but very short on convincing examples of either successful or mismanaged research.

Never has there been a greater need than now for a book on the effective management of an inherently labour-intensive activity. Never has there been greater need for advice on how to make maximum use of scientific talent, by example and inspiration, and by providing scientists with efficient tools and support. Yet I looked in vain for specific examples of people who have succeeded in making better use of a scientist's time, or examples of balanced (and unbalanced) research portfolios. Where, at a time when those responsible for granting or rejecting applications for research funds are so well aware that funds are flowing not to the most deserving areas of research but to scientists who can write a convincing proposal, is advice on how to put forward your case for research? What are the differences between a good and a bad research proposal? To be more chari-

Competent if not inspired

David Fishlock

Effective Management of Research and Development. By P. A. F. White. Pp. x+295. (Macmillan: London and Basingstoke, May 1975.) £10.00.

table, the author's most recent experience of research management, as a divisional head at the atomic weapons factory at Aldermaston, one of the most secret enclaves of science in Britain, has probably had an inhibiting effect on his readiness to supply or seek out the kind of details and examples that could have enlivened his book so much. But it scarcely excuses him from the charge of publishing out-of-date information. The long lists of research laboratories in Britain, given presumably to help the research manager with a problem to select a contractor who might help, contain names that no longer exist, and record budgets of four or five years ago, even for a public corporation such as the National Research and Development Council whose annual reports are readily available. One table offering the ratio of research funds spent in-house to those subcontracted, for 11 industries, is dated 1966-67.

When, however, the author drops the pretence of providing a handbook of up-to-date research data and concentrates on writing a textbook of research management, he stands on firmer ground. The latter half of his book starts with a chapter on the choice of a research and development portfolio—"probably the most important task of research management"—in which he sets out the principles, basically three, and reviews the sophisticated techniques which nowadays can help the manager to calculate economic benefit. Here I would cavil only at the brevity of the section on the stopping of research projects, acknowledged to be "one of the most difficult of a director's activities" yet commanding less than four pages when a whole chapter would have been fully justified. But it leads into further chapters on the control of projects, on efficiency and productivity in research and development, and on choosing research staff.

At the end of each chapter the author provides a brief but lucid summary of the points he has been trying to make. Rereading this review I think that the point I really want to make is that Mr White has almost totally neglected the human factor in an activity where, perhaps more than in any other field of endeavour, the difference in terms of success between competent and inspired research management—as between competent and inspired research—is a very big factor indeed. □

SINCE the previous volume in this series of symposia devoted to virology was published in 1968 progress in the field has been considerable. This compilation of 14 articles on the theme of control processes in virus multiplication is, therefore, an invaluable source of information for students and would-be students of animal virology.

Like other symposia in the series this is a collection of specially written review articles, rather than research papers, aimed at a readership of general microbiologists. This symposium deals principally with animal viruses. Only 4 of the 14 articles are concerned specifically with bacteriophages, and none of them considers the multiplication of plant viruses though there is a mention of certain structural aspects of these in an article by Showe and Kellenberger.

The editors have to a large extent succeeded in keeping the articles within

the stated theme. Reviews which are not so confined include the first three which are intended to provide back-

Latest virology

Control Processes in Virus Multiplication. (25th Symposium of the Society for General Microbiology, London, April 1975.) Edited by D. C. Burke and W. C. Russell. Pp. viii+449. (Cambridge University Press: London, New York and Melbourne, 1975.) £9.00; \$27.50.

ground information to the subsequent reviews. Indeed, Dressler in a very readable article admits that very little is known about the control of DNA replication but explains the advantages available to DNA molecules which adopt a circular form for replication, and goes on to describe the way the

T7 bacteriophage genome replicates without adopting a circular form.

Some of the articles are not easily assimilated perhaps because they each deal with a different aspect (or closely related aspects) of control for all the main classes of animal virus; they may have been easier to assimilate if each had dealt with a different class of viruses but had explored all aspects of control known within that class.

I strongly recommend this comprehensive collection of authoritative review articles on a theme of great significance in the field of virology, and will conclude with a quotation from the editors' preface: "... it has been our intention to describe the 'state of the art' at the time of writing, and if the reader is more impressed on occasions by our lack of knowledge, then we trust he or she may be stimulated to repair the deficiency!" **A. C. R. Samson**

Tropical landforms

Tropical Geomorphology: A Study of Weathering and Landform Development in Warm Climates. (Focal Problems in Geography Series.) By Michael F. Thomas. Pp. xii+332. (Macmillan: London and Basingstoke; distributed in USA and Canada by Halsted Press.) £2.95.

THE terms bornhardt and inselberg reflect the keen interest taken by German scientists in tropical landforms during the late 19th century. The British came in strongly in 1911 with J. D. Falconer's account of the geology of northern Nigeria. Comprehensive summaries appeared later, the chief being S. Passarge's *Panoramen afrikanischer Inselberg-landschaften* (1928) and Karl Sapper's remarkable *Geomorphologie der feuchten Tropen* (1935), and recently the French have produced much literature on tropical landscapes, including Jean Tricart's *Le modelé des régions chaudes, forêts et savanes* (1965) which was translated into English by C. J. K. de Jonge in 1972.

Now Dr Thomas, having published numerous percipient articles, has produced the first full treatment of tropical geomorphology written originally in English. He balances the results of his own wide-ranging field studies against the findings of other warm-climate geomorphologists, and summarises carefully the issues that are fundamental to an understanding of the development of tropical landforms and regoliths. In addition, by analysing in detail weathering processes and their relationships to common morphometry, he ensures that the reader understands geomorphogenesis anywhere under warm bioclimatic conditions in which the work of wind, frost and waves is absent.

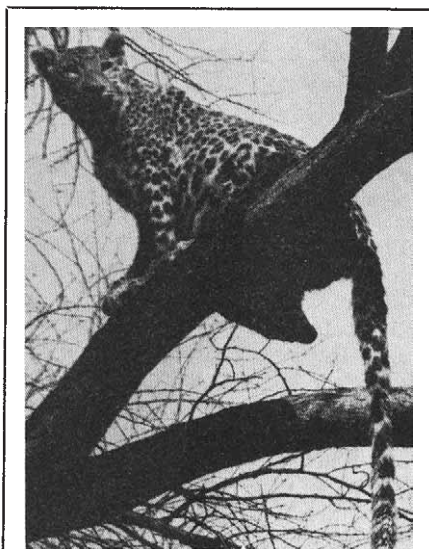
Part 1 contains five chapters on weathering and in these the author stresses the increased tropical rate of endothermic chemical reactions, the stability of vast horizontal surfaces, and the frequent occurrence of silicate minerals in rock exposures. The description of the processes are set out clearly and concisely, particularly the eluviation of silica and the formation of duricrusts and of deep-seated profiles.

Part 2 discusses the character and development of the tropical terrain and includes chapters on distinctive individual landforms, on hill-slopes, and on pediments and pediplanation. The text ends with a bibliography and three indexes.

Throughout, the author recognises that the processes undergo complex variations in intensity and that inherited or relict forms may be part of

the general picture. The landforms characteristic of the tropics are shown to experience a continuity of sub-aerial morphogenesis over a prolonged period during which interruptions by tectonic and climatic pulses are localised in space and time. Dr Thomas makes the reader aware of the many problems ahead and of the great progress made during the last few decades. His concise analysis combines clarity of exposition with clear-cut diagrams and ample quantification so successfully that it deserves to become straight-away the standard text on the geomorphology of the humid tropics.

Robert P. Beckinsale



Thereby hangs a tail: a highly placed leopard watches for prey. From *Tigerland*. By Kailash Sankhal. (Collins: London, May 1975.) £3.50.

Antibiotic agents

Mechanism of Action of Antimicrobial and Antitumor Agents (Antibiotics, vol. 3.) Edited by J. W. Corcoran and F. E. Hahn. Pp. xii+742. (Springer: Berlin and New York, 1975.) DM.188; \$77.10.

THIS volume is the third of a series and provides up to date information on many of the classes of antibiotics described in the first volume published eight years ago. In spite of the inclusion of 46 chapters, it is not a complete review and classes of antibiotic such as the actinomycins and nitrofurans, which are missing, will form the basis for a future volume. Among the 3,500 or so references are to be found a sprinkling of 1973 quotations, so the book is reasonably up to date, which is a good achievement for a publication of this complexity.

The book deals with antibiotics defined in the broadest sense of the word and chapters are included on alkaloids and synthetic chemicals which are

clearly like antibiotics in their mechanism of action. Descriptions are given of a great variety of agents which have properties ranging from antibacterial and antiparasitic, to antitubercular and antitumour, and agents which are primarily useful as laboratory probes for investigating biological processes are also discussed. Indeed, some chapters contain accounts of chemicals which seem to have no practical value and which, presumably, are still looking for some disease to cure.

A clear sign of the emerging interest in antibiotics as antitumour agents is evident from the first section of the book on agents that interfere with nucleic acid biosynthesis. The majority of the compounds described in this section have some antitumour properties, and though only bleomycin and members of the anthracycline group are of proven clinical value, it is apparent that this is an area in which new discoveries may well be made. A second section deals with agents that interfere with protein biosynthesis and a miscellaneous final section is devoted to agents that interfere with specific enzymes and with membrane or cell wall biosynthesis and with agents whose mechanism of action is unknown.

The editors have obviously given a lot of thought to the design of the book and to the selection of authors. What emerges is a pleasingly uniform series of chapters dealing with the mechanism of action of various antibiotics. With few exceptions, the chapters are authoritative and provide critical accounts rather than just a catalogue of reported work.

Mechanisms of selectivity are discussed in a few of the chapters. Ethidium, for example, is an antiparasitic agent probably because it interacts specifically with closed circular molecules of extrachromosomal DNA. The characteristic sensitivity of squamous cell carcinoma to bleomycin is explained by the fact that this tumour type concentrates the drug and is also unable to convert it to its non-toxic form. Unfortunately, many authors, having described the probable mechanism of action of a compound, make no effort to explain the basis of its selectivity. Why, for instance, should one intercalating agent be an antimalarial, another antitumour, and yet another just a poison? Perhaps the reasons for selectivity are still little understood, but a few comments by each author, even if only of a speculative nature, would have rounded off a good book.

All in all this is a fine volume which, combined with the promised volume dealing with agents not covered in this book, provides a comprehensive and critical survey of our present day knowledge of antibiotics and related compounds.

T. A. Connors

Natural and Synthetic Polymers: An Introduction. By Henry I. Bolker. Pp. xiv + 688. (Dekker: New York, November 1974.) \$29.75.

THE great increase in the number and type of synthetic polymers during the 25 years since the publication of K. H. Meyer's book under almost the same title as this one, and the great deal more that we now know about both synthetic and natural polymers, would make it a daunting task for anyone to follow precisely in Meyer's footsteps. Dr Bolker does not attempt to do so. Instead, he maintains wide coverage by making only brief statements about many of the polymers with which he deals, each of which could often be expanded into a whole book in itself; though, with his commendable conciseness, he manages to pack a surprisingly large amount of information into a small space.

His account differs from others I have read in basing the sequence in which the polymers are considered, not upon their chemistry or function, but upon the molecular geometry of their structure, passing from linear through branched to cross-linked architectures and, therefore, from simple to complex. This gives some natural polymers somewhat awkward neighbours but it has a logic about it which is appealing, which leads students gently by the hand (and this is why the book was written), and has a great advantage in bringing together in a unified way matter normally taught separately by 'natural product' chemists, by polymer chemists and by biologists. The upshot is a very readable book.

Linear polymers are dealt with in four chapters of which the longest (and the longest in the book) is the first, dealing with cellulose as the type linear homopolymer upon which polymer chemistry was for so long based. The discussion of branched polymers, also covered in four chapters, includes considerations of heteropolysaccharides and nucleic acids as well as the multitudinous synthetic copolymers. Cross-linked polymers include proteins (and the genetic code) and lignin. For each group of polymers, attention is paid to structure, to properties, to synthesis or biosynthesis, and to the use by man or the function in nature. Each chapter carries its own reference list and many of the great names of polymer science will be found here though the almost completely chemical approach has meant that some of the non-chemists are, sadly, not mentioned.

In the sections covering those aspects about which I know best the book is not up-to-date—the explosion of knowledge of polysaccharide structure during recent years, for instance, is hardly touched upon—but it would be surprising if every part of a book with such wide

coverage could be 'stop press'. I personally would have been happier to have had more diagrams of the ball-and-spoke type rather than chemical formulae; they give a better appreciation of the three-dimensional geometry. And some of the diagrams could have been drawn more tidily. These are, however, minor criticisms of a book which skilfully takes students through a complex series of discoveries, which turns a maze into a garden path. It is to be recommended strongly to students not only of polymer chemistry but of physics and biology, and indeed mature scientists in these disciplines might profit from reading it. I have certainly profited myself. **R. D. Preston**

Polymers

Ionic Polymers. (Materials Science Series.) Pp. xii+416. Edited by L. Holliday. (Applied Science: London, 1975.) £14.00.

AFTER reading this book I not only share the editor's view that materials like the inorganic borates, phosphates and silicates should be regarded as polymers of a particular (ionic) type, I feel that the very existence of the book will make it impossible for authors of future texts on polymer chemistry to resist the inclusion of these inorganic compounds in what is clearly their proper context. The text, taken in order, gradually passes from comparatively orthodox—in polymer science terms—discussion of the synthesis and properties of certain predominantly organic macromolecules to descriptions of the sheet-like and three-dimensional structures of the inorganic polymers. I am left with more than a sneaking suspicion that if history had permitted organic polymer chemistry to precede the study of the silicates, the intentional preparation of the latter would have been hailed as a substantial achievement in polymer synthesis.

The eight chapters, taken together, give a pretty comprehensive view of ionomers, from detailed structural studies of the group to their commercial utilisation. The first chapter provides a general discussion, dealing with nomenclature and general properties. After that the text covers ionomers of the thermoplastic and elastomeric types and moves on through a range of materials of increasing ionic content to the longer standing polyelectrolytes and inorganic silicates, and so on. In the middle range, cements and soil-conditioning materials are found together with the metal dicarboxylates, for which the name 'halatopolymers' is coined—are they salts or polymers, or both?

Naturally, the various articles vary

in approach but the important thing is the book as a whole: it not only fills a void in the literature of polymers, it also teaches a valuable lesson which should leave its mark on all future contributions.

A. D. Jenkins

Molecular Behaviour and the Development of Polymeric Materials. Edited by A. Ledwith and A. M. North. Pp. 553. (Chapman and Hall: London, February 1975. Distributed in the USA by Halsted Press.) £12.00.

AT the research level a large proportion of new books consist of collections of review chapters, contributed by different authors. Where such collections are aimed at the specialist in a particular discipline, they are easily judged by the standards of that specialist. The present volume is more difficult to judge, since it has a different motivation, being compiled as a richly deserved tribute to Professor C. E. H. Bawn, on his retirement from the University of Liverpool. The review articles which form the book have been written by friends and former colleagues of Professor Bawn and their only other unifying theme is that they all lie within the boundaries of polymer science. The result is a somewhat heterogeneous book with topics that cover a very wide range of interests.

The 14 chapters comprise three reviews of polymerisation mechanisms, four broad reviews of specific polymer types (polybutadienes, other elastomers, polyolefins and polyurethanes), two reviews on the chemical reactions of polymers and five reviews on the structure and physical properties of polymers. All of the chapters have been written to a high standard and editorial monitoring has been good. I found the chapter by Small, on polyolefins, and the chapter by Ledwith and Sherrington, on polymers as catalysts, especially interesting, the former for its critical discussion of why so few polyolefins are commercially successful and the latter for its treatment of an underdeveloped area of great potential.

This is a book which is unlikely to be read by the specialist looking for new insight into his own discipline. Certainly in those areas where I can claim expertise, there is little which has not already appeared elsewhere. But for those wishing to get a feel for what is going on in a selection of other areas of polymer science this volume will provide much of interest. At the price (which is high but not excessive) few individuals will find enough of sufficiently urgent interest to wish to buy a personal copy but the book can be highly recommended as a library purchase.

N. C. Billingham

Understanding of the developing child

Learning and the Development of Cognition. By Barbel Inhelder, Hermine Sinclair and Magali Bovet. Translated by Susan Wedgwood. Pp. xiv+308. (Routledge and Kegan Paul: London, February 1975.) £4.95.

PIAGET'S theory is frequently presented as a rigid maturational system in which stage follows stage, more or less regardless of the child's social milieu, so that specific training procedures to speed up intellectual development are ineffective and pointless: either the child has the developmental structures to assimilate the material, in which case nothing substantially new can be acquired, or else those structures are not yet developed and no useful learning can take place.

No wonder that psychologists and educators alike, in their efforts to understand and to instruct the developing child, recoil from this altogether pessimistic picture and put it aside as irrelevant. No wonder, either, that any experimental evidence of successful intervention programmes in teaching mental operation at a younger age than commonly expected is flaunted as proving Piaget's theory wrong. Fortunately, the interpretation I have mentioned is but a caricature of the theory, born out of the unquestioned assumption that between a hereditarily given, unchanging potential and learning in the form of storing and coding environmental information there are no other factors contributing to the growth of intelligence. Piaget rejects both extremes of the heredity-environment controversy without lapsing into an interactionist stance in which one is locked in interminable arguments about the correct statistical proportions, but by proposing mental development as a psychological process altogether different from the learning of information or the unfolding of instinctual patterns.

This book, if nothing else, is a reminder that Piaget's theory is not at all aversive to a study of environmental input as it affects children's efforts at understanding their world. On the contrary, the investigations reported are predicated on the idea that developmentally suitable encounters will bring about intellectual progression; yet there is no concomitant desire of speeding up, rather of observing the developmental process in a concrete setting. The authors, who are Piaget's closest collaborators of many years, conceptualise this progression as a construction on the part of the child in the face of

fluctuations and conflicts between apparently divergent schemes of thinking. The encounters are devised precisely to expose children, if possible, to an awareness of this conflict and thus to bring about a restructuring of these schemes; in other words, a progression from a base of less or conflicting understanding to a stage at which the conflicting views are subsumed as non-disturbing facets of a more adequate understanding. As an illustration, in the comparison of a W-shaped line and a straight line, the length of the lines and the number and configuration of segmental units making up the lines can create a conflict in a child who does not fully coordinate the concept of length in relation to segmentation and unit element.

In all experiments the children were carefully selected as being in a transitional phase and tested before and after training; each child's performance was individually analysed and tentative categories of progress—or lack of it—were proposed. Though the methods of experimentation include the use of such familiar concepts as assessment of the quantity of a liquid, numbers, inclusion, conservation of substance and weight, the thinking activities to which the children were exposed are ingenious and original, not only in helping the child's progress, but also in helping us to understand and observe the process of development. In the foreword Piaget singles out some of the more important theoretical insights to be gleaned from this research which is frankly acknowledged to be but an exploratory beginning in the search towards the unravelling of the process of development.

Thus the book is another milestone in Genevan research and contains the clearest exposition so far of Piaget's elusive concept of developmental equilibration. One may add to this that the translation was supervised by one of the authors and is not full of mistranslations as are, unfortunately, many other works on Piaget's theory. But make no mistake, like other Genevan writings it is not easy reading and it does have weaknesses for which the Genevans can be so easily criticised: points of empirical and methodological inadequacy, incomplete protocols, theoretical overinterpretations. A reader who cannot see beyond these will no doubt be dissatisfied. But those who want to understand better the nature of environmental occasions leading to intellectual development, and who search for guidelines in worthwhile psychological research will find here an exciting theoretical framework, all the more so on account of its obvious practical and educational applications.

H. Furth

Introduction to Marine Geology and Geomorphology

CUCHLAINE A. M. KING

During the ten years since the first edition of *Oceanography for Geographers* (distributed in the U.S.A. as "Introduction to Oceanography") was published, there has been an explosive development in nearly all aspects of the subject. So much has been written concerning this aspect of oceanography that it has been necessary for the author to prepare the new edition of *Oceanography for Geographers* as two separate books, both of which are almost entirely new. This is the first.

Cloth £9.90 Paper £4.90

Introduction to Physical and Biological Oceanography

CUCHLAINE A. M. KING

The physical aspects considered in this book include an introduction to the character of ocean water and its circulation in the form of surface currents and deep wave movements. The subjects of tides and the various waves that disturb the surface of the ocean are discussed and the biological aspects of oceanography form a major and topical theme through the second half of the volume.

Cloth £11.00 Paper £5.50

Rural Recreation in the Industrial World

I. G. SIMMONS

The provision of rural recreational facilities in industrialized societies is of growing economic and social significance. This book deals with the demand for outdoor recreation in industrial nations in the northern hemisphere during the last twenty years and the response to it in the development of facilities by public and private enterprise.

Cloth £9.95

The Ecology of Natural Resources

I. G. SIMMONS

... good books require no apologies whatever for their subject... why is his book so intellectually satisfying when others merely depress? One factor is the sense of order so crucial in a textbook... Far from making this a dull academic tome, it brings welcome relief from the ultimately more tedious Ehrlich-like stridency of many of Simmons's predecessors.' *New Society*

Cloth £8.00 Paper £3.85

Edward  Arnold

25 Hill Street, London W1X 8LL

obituary

Joseph Hewitt, designer of the Hewitt satellite camera, died on May 21 at the age of 63.

Born at Leicester in 1912, Hewitt took a first degree in Botany at University College, Leicester in 1933, but after some research on the colorimetry of cells, he became convinced that optics was his *métier*, and he took a postgraduate course on technical optics at Imperial College, London in 1939. In 1942 he joined what is now the Royal Radar Establishment at Malvern, where he remained for the rest of his career, becoming leader of the photographic unit and also working on lasers. Hewitt will be particularly remembered as the designer of the Hewitt camera, a Schmidt-type instrument of 600 mm aperture with a 900 mm mirror and a field-flattening lens. Two of these cameras were made and are at present owned by the Ordnance Survey, for whom Hewitt acted as consultant for the past three years, after retiring from

RRE in 1972. The cameras have proved to be the most accurate in the world for observation of artificial satellites, and the observations have been extensively used in geodetic and geophysical researches.

Sir William Hodge, FRS, ScD, FRSE, who was Lowndean Professor of Astronomy and Geometry and Master of Pembroke College, Cambridge, from 1958–70, has died at the age of 72.

Born at Edinburgh and educated at St John's, Cambridge, Sir William spent most of his working life in Cambridge, with the exception of five years as a lecturer at Bristol and a year at Princeton. After holding a research fellowship at St John's he became a staff fellow of Pembroke, until in 1936 he became Lowndean Professor. In 1958 he was elected Master of Pembroke, and, together with Lady Hodge, did much to preserve a friendly atmosphere in the

college. Hodge's main mathematical contributions were in the field of algebraic geometry. His theory of harmonic integrals has had a profound influence on the development of geometry in the past 30 years. After the war he played a leading role in the formation of the International Mathematical Union, serving as vice-president from 1954–58. He was one of the instigators of the British Mathematical Colloquium, and was also president of the London Mathematical Society, the Mathematical Association and the Cambridge Philosophical Society. He was elected a fellow of the Royal Society at the early age of 35, and was physical secretary from 1957–65 and a vice-president from 1959–65. Recognition for his public services came in 1959 when he was knighted. The Royal Society awarded him its Royal Medal in 1957 and the London Mathematical Society gave him the de Morgan Medal in 1959.

announcements

Awards

The 1975 **Glaxo Travelling Fellowships** for British science writers have been awarded to **Dr and Mrs Oliver Gillie** (joint award), **Michael Barnes** and **Frank Walsh**.

Appointments

W. Ashworth has been appointed pro-vice-chancellor of the University of Bristol.

W. F. Wood has been appointed dean of the faculty of environmental studies at Heriot-Watt University.

Miscellaneous

Vision research. Further information about the research fellowships being offered by the Worshipful Company of Spectacle Makers, may be obtained from: The Clerk of the Company, Mr C. J. Eldridge, Apothecaries' Hall, Blackfriars Lane, London EC4V 6EL, UK.

International meetings

September 21–25, **Mammalian cell culture and its application**, Birmingham, Alabama (Professor R. T. Action, Symposium Chairman, Department of Microbiology, University of Alabama in Birmingham, University Station, Birmingham, Alabama 35294).

September 21–26, **Electrochemistry in non-aqueous solutions**, Baden, Austria (26th ISE Organising Committee, Verein Österreichischer Chemiker, Eschenbachgasse 9, A1010 Vienna, Austria).

September 21–27, **Space and energy**, Lisbon, Portugal (International Astronautical Federation, 250 Rue Saint-Jacques, 75005 Paris, France).

September 22–24, **Engineering in the ocean environment**, San Diego (Institute of Electrical and Electronics Engineers, Technical Activities Board, 345 East 47th Street, New York, New York 10017).

September 22–24, **Aerospace simulation facilities**, Ottawa (E. S. Hanff, National Research Council of Canada, Montreal Road, Ottawa, Canada K1A 0R6, Canada).

September 22–24, **Turbulence in liquids**, Missouri (Professor G. K. Patterson, Chemical Engineering Department, University of Missouri-Rolla, Rolla, Missouri 65401).

September 22–25, **Origins and evolution of language and speech**, New York (Conference Department, The New York Academy of Sciences, 2 East 63rd Street, New York, New York 10021).

September 22–26, **Microdosimetry**, Verbania Pallanza, Italy (Secretariat of the 5th Symposium on Microdosimetry, Commission of the European Communities DG XII, Biology Division, Dr H. G. Ebert, 200 rue de la Loi, B1040 Brussels, Belgium).

September 22–26, **Ferroelectricity**, Zurich (Secretariat EMF3, Zurich

Laboratory of Solid State Physics, Swiss Federal Institute of Technology, HONGERBERG CH8049, Zurich, Switzerland).

September 22–26, **Vertebrate palaeontology and comparative anatomy**, Edinburgh (S. M. Andrews, Royal Scottish Museum, Chambers Street, Edinburgh EH1 1JF, UK).

September 23–25, **Cybernetics and society**, San Francisco (L. S. Coles, Artificial Intelligence Center, Mento Park, California 94025).

September 23–25, **Instrumentation in oceanography**, Bangor, Wales (Conference Department, Institution of Electronic and Radio Engineers, 8–9 Bedford Square, London WC1 3RG, UK).

September 24–25, **Regulation of biosynthesis of bacterial walls and membranes**, Newcastle upon Tyne (The Meetings Assistant, Society for General Microbiology, Harvest House, 62 London Road, Reading, Berkshire RG1 5AS, UK).

September 24–26, **Polymer physics**, Shrivvenham, UK (Mrs H. Higdon, Physics Department, Brunel University, Uxbridge, Middlesex UB8 3PH, UK).

September 25, **Electron spin resonance bioenergetics**, London (Dr J. Barber, Botany Department, Imperial College, London SW7, UK).

September 29, **Chromatin**, Glasgow (Dr A. J. MacGillivray, Beatson Institute for Cancer Research, 132 Hill Street, Glasgow G3 6UD, UK).

September 30–October 6, **Biometeorology**, Israel (Mr Y. Lumas, Department of Agricultural Meteorology, Meteorological Service, PO Box 25, Bet-Dagan, Israel).

Reports and publications

Great Britain

Annual Report of the Grassland Research Institute for 1974. Pp. viii + 163. (Hurley, Maidenhead, Berkshire: The Grassland Research Institute, 1975.) £2. [26]
Report of the Tropical Products Institute, 1972/1974. Pp. v + 96 + 12 plates. (London: Tropical Products Institute, Publications Section, 127 Clerkenwell Road, EC1, 1975.) 90p net. [26]
Medical Research Council. Criteria for Controlling Radiation doses to the Public After Accidental Escape of Radioactive Material. Pp. vii + 48. (London: HMSO, 1975.) £1.30 net. [26]
Home-Grown Cereals Authority. Progress Reports on Research and Development, 1973/1974. Pp. 23. (London: Home-Grown Cereals Authority, Hamlyn House, Highgate Hill, N19, 1975.) [26]
The Work of the Rutherford Laboratory 1974. Edited by J. R. Smith and F. M. Telling. (Science Research Council.) Pp. 207. (Chilton, Didcot: Science Research Council, Rutherford Laboratory, 1975.) [46]
Avon Council on Alcoholism. Fifth Annual Report 1975. Pp. 31. (Bristol: Avon Council on Alcoholism, 1975.) 40p. [56]
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